Spatial, Temporal and Mechanistic Characterization of Apoptotic Death in the Developing Subventricular Zone

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ABSTRACT

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The neonatal subventricular zone (SVZ) is a site of continued postnatal neurogenesis, and is the source of cortical glial cells. Apoptosis is an endogenous process of cell destruction, and is a key event in the proper development of the SVZ. Despite its importance, there is still a lack of knowledge regarding the temporal and spatial occurrence of neonatal SVZ apoptosis, cell types affected and the underlying intrinsic and extrinsic mechanisms that guide the process. This thesis addresses these issues, and in addition, finds a nontraditional mode of neurotrophic action for cell survival in the neonatal SVZ.

We assessed SVZ apoptosis by subregion, employing the cell death markers, pH2ax and cleaved caspase 3. The medial SVZ contained the highest density of dying cells at p0, while at p7 there was no significant difference in the apoptotic cell density distribution in the SVZ subregions. Combining cell type specific markers with the death markers used, revealed immature postmitotic neurons were the primary cell type cleared in the p0 medial SVZ. The majority of dying cells in the p7 dorsolateral SVZ (SVZdl) were unable to be identified. Using stereotactic injection of a GFP expressing lentivirus, we determined the p0 medial SVZ cell population to be migratory cells bound for the olfactory bulb.

An investigation into the intrinsic and extrinsic mechanisms mediating cell death in the neonatal SVZ, showed BH3-only protein Bim expression in the p0 and p7 SVZ, as well as significantly decreased p0 medial SVZ apoptosis in Bim knockout mice. Bim knockout mice did not show a significant change in apoptosis in the p7 SVZdl. TrkB knockout mice have shown a survival role for the receptor in the lateral ganglionic eminence of the neonatal SVZ. To test this in the p0 medial SVZ using a more specific method, a TrkB blocking antibody was injected into the p0 medial SVZ. This resulted in a significantly higher number of apoptotic cells in the p1 medial SVZ versus controls.

These studies demonstrate the dynamic nature of the SVZ with its changing density and identity of apoptotic cells within the subregions. It has also shown the influence of Bim and TrkB signaling in neonatal SVZ apoptosis and survival. Finally, it has identified a premigratory cell population in the p0 medial SVZ, whose survival is mediated by neurotrophin signaling at their site of origin.

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LIST OF ABBREVIATIONS AND ACRONYMS

AD	Alzheimer's Disease
Apaf-1	Apoptosis Protease-Activating Factor 1
BDNF	Brain Derived Neurotrophic Factor
Bcl-2	B-cell lymphoma-2
BLBP	Brain Lipid Binding Protein
CNS	Central Nervous System
DR	Death receptors
DISC	Death Inducing Signaling Complex
DRG	Dorsal Root Ganglion
FADD	Fas-associated death domain
GFAP	Glial Fibrillary Acidic Protein
ISEL	In-situ end labeling
JNK	Jun N-terminal kinase
LV	Lateral Ventricle
MCM2	Minichromosome Maintenance Protein 2

NGF	Nerve Growth Factor
NT	Neurotrophin
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
OB	Olfactory Bulb
P	Postnatal Day
PD	Parkinson's Disease
PDGFRα	Platelet Derived Growth Factor Receptor α
pH2ax	PhosphorylatedhistoneH2ax
PUMA	p53 upregulated modulator of apoptosis
PNS	Peripheral Nervous System
pTrkB	PhosphoTrkB
RGC	Radial Glial Cell
RMS	Rostral Migratory Stream
SVZ	Subventricular Zone
SVZa	Anterior SVZ
SVZdl	Dorsolateral SVZ
SVZge	Lateral SVZ
SVZspt	Septal SVZ
TNFR	Tumor Necrosis Factor Receptor
TUNEL	Terminal deoxynucleotidyltransferasedUTP Nick End Labeling

VZ	Ventricular Zone

ACKNOWLEDGEMENTS

First and foremost I would like to thank my thesis advisor, Dr. Lloyd Greene. Lloyd has taught me so much about the process of asking the right scientific questions and the critical thinking necessary to arrive at the answers. He is a brilliant mind, but more importantly a fair and honest person. I could not have asked for a better mentor.

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Finally, I would like to thank my friends and family for all their love and support. To my friends on the P&S 15th floor, at Columbia and beyond Morningside Heights, thank you for making the past 6 years a blast. I appreciate your friendship so much. My mother is the reason I am able to be where I am today. I would not have completed my PhD without her. She has stood by me throughout this long process, patiently listening and motivating me. I dedicate this work to her and my late grandmother with all my love.

DEDICATION

For Mom and Mama Pacing

Chapter 1. General Introduction

1.1 Apoptosis

1.1.1 Apoptotic Pathway

Apoptosis is a key event in the maintenance of physiological equilibrium in the organism. The apoptotic cell death process has variety of important roles, namely in development, the stress response, cell proliferation and cell population survival [1]. Apoptosis can be described as the elimination of a cell through either an intrinsic or extrinsic cell signaling pathway, that physically manifests itself in cellular blebbing and shrinkage, as well as the condensation and fragmentation of the cell nucleus [2]. Necrosis and autophagy are commonly associated with apoptosis under the umbrella term, programmed cell death. Like necrosis and autophagy, apoptosis has been reported to occur in pathological conditions. What differentiates apoptosis is the fact that it is also an essential process in healthy tissue as a part of the normal development and maintenance of the organism, and occurs without causing an inflammatory response. Apoptosis can also take place without the activation of lysosomal enzymes, unlike autophagy [3].

The discovery and better understanding of apoptosis is the result of an evolution of scientific techniques and the continuous progress built from the work of many investigators. Apoptosis was first noted by Hippocrates, yet it was not until the 1970s that this biological event was more formally recognized [4]. The advancement of the concept of apoptosis was largely due to the contributions of John Kerr. Kerr, while researching his PhD thesis, noticed a unique histological appearance of dying cells in

the liver following interruption of the portal vein branches [5]. These dying cells, with their unique cellular morphology, did not fall within the classification of necrotic cells. The distinct appearance of this dying cell population thrust the idea of a new class of cell death forward. Kerr expanded his findings with electron microscopy images of the dying cells and observations of this type of cell death in pathological and healthy tissue. [5].

Kerr's morphological characterization of apoptosis was extended with experiments that described what was occurring biochemically within the cell. The work of Tata revealed the RNA and protein synthesis requirement for apoptosis in the elimination of the tadpole tail [6]. Other studies have gone on to confirm Tata's findings, showing the necessity of RNA translation and protein expression in a range of cell types such as thymocytes and cerebellar granule neurons [7, 8] Yet, when apoptosis was induced in a human promyelocytic cell line by treatment with cyclohexamide, a protein synthesis inhibitor, protection from cell death was not obtained [9]. The independence of apoptosis from RNA and protein synthesis has been demonstrated in other cells types, such as T lymphocytes, supporting the idea that the requirement for RNA and protein synthesis may be dependent on the cell type and biological context.

Twenty years had passed from the initial characterization of apoptosis to the first experiments that pinpointed the genes involved in the process [10]. Studies performed in *Caenorhabditis elegans* (*C. elegans*) identified the key apoptotic genetic players. Horvitz and colleagues found a decrease in the amount of dying cells in *C. elegans* mutants that had the genes ced-3 and ced-4 deleted [11]. By the early 1990's, the death

genes ced-3 and ced-9 characterized in *C. elegans* were found to have their counterpart mammalian homologues, the interleukin-1 beta converting enzyme and the proto-oncogene product Bcl-2 family, respectively [12, 13].

Identification of the various proteins involved in apoptosis ultimately led to a description of the larger pathway that encompassed all these molecular components. The intrinsic and extrinsic pathways are the two main apoptotic cell signaling pathways. These signaling cascades differentiate themselves from one another by involving unique initiators and different molecular components, but converge on the activation of caspases and the clearance of the cell. The two cell death pathways also undergo crosstalk via the activity of the BH3-only protein, Bid. A full description of these two pathways will be discussed in the following paragraphs.

The extrinsic pathway describes apoptosis activation via the binding of specific ligands, such as cytokines to death receptors (DR) on the surface of the cell. These receptors, including DR4, DR5 and Fas, are part of the Tumor Necrosis Factor Receptor (TNFR) superfamily [14]. Following activation of these receptors is the formation of the death inducing signaling complex (DISC), which arises from the recruitment of various protein components, particularly, the adaptor Fas-associated death domain (FADD), caspase 8, and a regulator of caspase 8 activity FLIP [15].

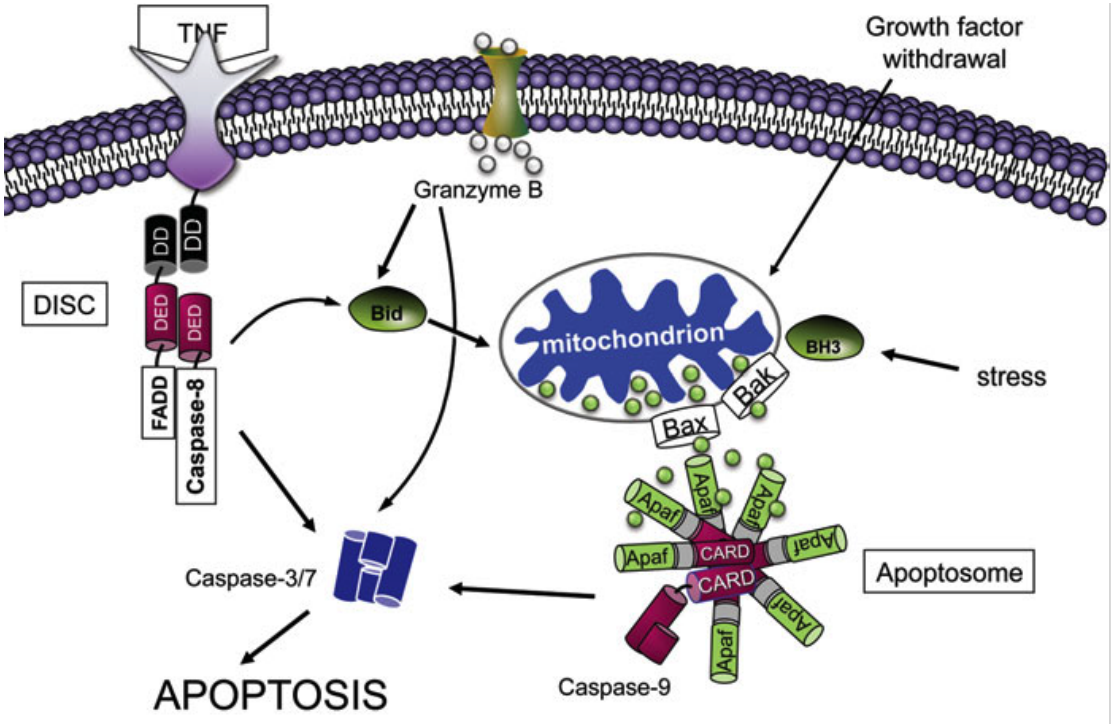


Figure 1-1. Extrinsic and Intrinsic Apoptosis Pathway. The extrinsic pathway, featured on the left, is activated by an extracellular ligand binding to a death receptor embedded in the cellular membrane. In contrast, the intrinsic or mitochondrial apoptosis pathway on the right shows the involvement and reliance on the mitochondria in executing cell death [16].

As seen in Figure 1-1, caspase 8 serves an important role in carrying out apoptosis in the extrinsic pathway. Caspase 8 acts as an initiator caspase by starting the apoptotic cascade and cleaving downstream caspases [17]. It is these downstream caspases, caspase 3 and 7, which bind and cleave essential cell organelles for proteolysis, leading to cell death. It is also at this point that the two apoptotic pathways can converge to result in apoptosis.

The other cell death signaling process is the intrinsic apoptosis pathway. The intrinsic apoptotic pathway primarily involves the B-cell lymphoma-2 (Bcl-2) protein family and the mitochondria in the execution of apoptosis. The intrinsic signaling pathway is closely associated with developmental apoptosis and death following cellular stress [18]. This pathway will be the primary focus of this thesis.

As mentioned, the Bcl-2 proteins play an integral role in the intrinsic pathway. Studied for its implication in oncogenesis, the link between Bcl-2 and apoptosis was shown when overexpression of the protein protected against cell death [19]. The Bcl-2 protein family has expanded since then. Members are divided by falling into either the antiapoptotic (Bcl-x_L, Bcl-w, A1, Mcl-1, Boo/DIVA/Bcl2-L-10, Bcl-B) or proapoptotic group (Bax, Bak, Bok/Mtd, Bcl-x_S, Bcl-G_L and BH-3 only proteins) [20].

When a cell is presented with a death-inducing stimulus, the BH3-only proteins are the first Bcl-2 family proteins to act. These class of proteins share only the BH3 motif, the amino acid sequence LXXXGD (X represents any amino acid), amongst themselves and the rest of the Bcl-2 family [19]. The BH3-only proteins are solely proapoptotic and act to bind and neutralize antiapoptotic Bcl-2 proteins. Bik/Nbk, Bik, Bad, Hrk/DP5, Bid, Bim/Bod, Noxa, PUMA/Bbc3, Bcl-G_s and Bmf comprise the mammalian BH3-only family, and use their alpha helical BH3 domain to bind to the groove created by the BH1 and BH2 domains on the surface of antiapoptotic Bcl-2 proteins [20].

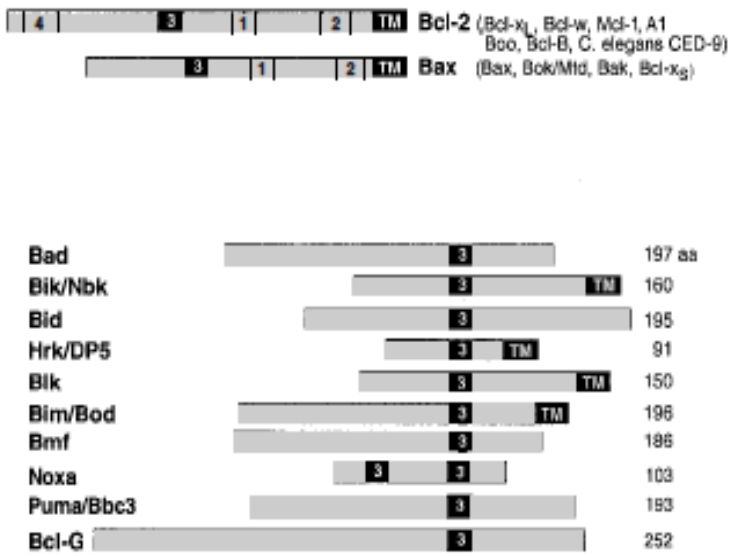


Figure 1-2. Structural comparison of mammalian Bcl-2 proteins [20].

Inhibiting antiapoptotic Bcl-2 proteins leads to the compromise and permeabilization of the mitochondrial membrane. A pore is formed on the surface of the mitochondria from the interaction of the Bcl-2 proteins, Bak and Bax. BH3-only proteins can also act at this stage. Bim, p53 upregulated modulator of apoptosis (PUMA), and Bid have been shown, in addition to their ability to inhibit antiapoptotic Bcl-2 proteins, to directly activate Bak and Bax [21, 22]. Triple knockout mice for Bim, Bid and PUMA exhibit an identical phenotype to double knockout mice for Bak and Bax, i.e. reduced cellular apoptosis, the presence of interdigital webs, and imperforate vaginas [21]. Future experiments will look at the conditions that favor direct or indirect activation of Bak and Bax by BH3-only proteins.

The changes in the mitochondrial membrane produced by the Bax-Bak pores culminate in the release of cytochrome c, a soluble protein important in the

mitochondrial respiratory chain, into the cytoplasm [23]. The presence of cytochrome c in the cytoplasm leads to the formation of the apoptosome. The apoptosome forms from cytoplasmic cytochrome c binding to apoptosis protease-activating factor 1 (Apaf-1), in the presence of ATP [24]. The apoptosome complex is essential for the activation of the caspases in the intrinsic apoptotic pathway. The apoptosome activates caspase 9, an initiator caspase. Caspase 9 cleaves and activates downstream effector caspases, such as caspase 3, similar to the activity of caspase 8 in the extrinsic apoptosis pathway.

The components of the intrinsic and extrinsic apoptotic pathway have been reviewed in this section. The pathways are isolated from one another until they converge in the cleavage and activation of the effector caspases. It was later discovered that the pathways had yet another point of overlap. In 1998, the work from two independent laboratories showed the activation of Bid by caspase 8 [25, 26]. Once activated, Bid translocates into the mitochondria and leads to the release of cytochrome c and eventual apoptosis. The existence of two unique and non-mutually exclusive apoptosis pathways adds to the complexity of apoptosis as a biological process.

1.1.2 Developmental Apoptosis

The notion that cell death is tightly linked with the normal development of an organism may appear counterintuitive. One would reason that more cells are needed, not less, during a period of growth. Experimental results, in which apoptosis is blocked, provide the strongest evidence for the importance of cell death in development. Apoptosis has been shown to be responsible for (1) sculpting, (2) deleting unnecessary

structures, (3) controlling cell numbers, and (4) eliminating abnormal, ectopic, or compromised cells.

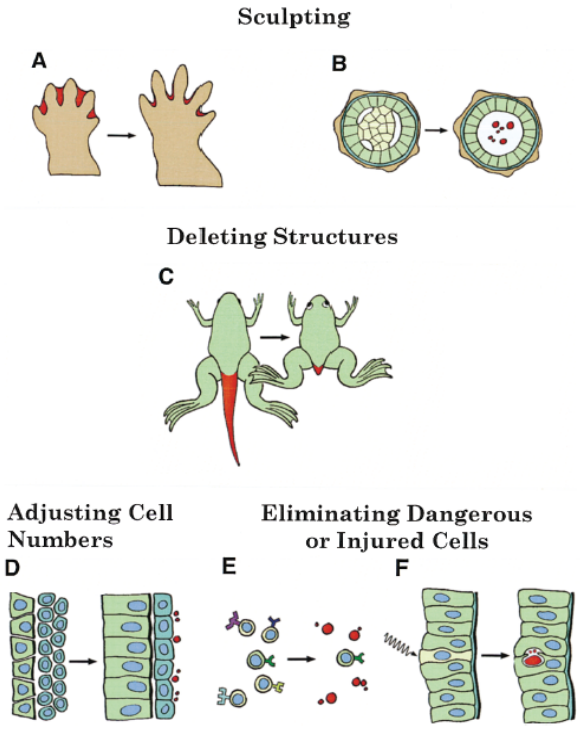


Figure 1-3. Functions of apoptosis include sculpting, deleting unnecessary structures, cell number adjustment and elimination of damaged cells [10].

Developmental apoptosis functions in the definition and shaping of appendages, and is based on the concept that cells are overproduced. There are an excess number of cells, more than what is required for functional purposes, and it is necessary for them to be eliminated. The postulated advantage for cellular overproduction is that it allows for greater flexibility, for structures to adapt to the needs of the organism at a particular time or environment [27]. A classic example is the removal of interdigital webs in most

mammals during the course of development. In Bax/Bak double knockout mice, the amount of apoptosis decreases and the interdigital webs are maintained in the animal [21].

In some cases whole structures are lost via apoptosis. The Mülleran duct gives rise to the uterus and oviduct, and is removed in males [27]. A similar mechanism occurs in female sexual organ development. The Wolffian duct, which is necessary for male reproductive development, is removed in females also via apoptosis [28]. Apoptosis is not only relevant in sex determination, but is also important for the physical changes that occur in the maturation of the organism. One of the most widely known examples is the elimination of the tadpole tail in the juvenile frog before it reaches adulthood.

Apoptosis also acts to control cell numbers. This function of apoptosis highlights the close relationship between proliferation and cell death. Studies in mice in which caspase 3 and caspase 9 have been individually eliminated show excess cells in the nervous system and death occurring perinatally [28, 29]. The extent of cell overproduction in the caspase 3 knockout mouse is demonstrated in the cortex, which exceeds the size of the skull. Another key example of apoptosis acting in the regulation of cell populations is in the death of lymphoid cells after host infection. T lymphocytes are overproduced during pathogen invasion. Some T lymphocytes remain, but a significant portion is cleared following the infection [30].

Finally, apoptosis can work to eliminate compromised or malfunctioning cells. Cells that have acquired DNA damage during proliferation or due to irradiation are

cleared via apoptosis [10]. Experiments in mouse nervous tissue that have proteins essential for DNA repair pathways disrupted, show an increase in DNA damage-induced apoptosis [31]. Apoptosis is not limited to damaged cells; ectopic cells are also removed through this process.

The examples discussed emphasize the functional importance of apoptosis. Nowhere is this clearer than in the nervous system. Many of the studies showing the necessity of apoptosis in development come from observations in nervous tissue [29, 30]. Surprisingly enough, the extent and implications of neuronal cell death were debated and argued for many years before its eventual acceptance as an essential component of normal brain development.

It has been estimated by studies done in the peripheral nervous system (PNS) that roughly 50% of newborn neurons are removed via apoptosis [32, 33]. One of the most widely studied systems for PNS apoptosis is the dorsal root ganglion (DRG). The DRG neurons in the upper cervical and thoracic levels undergo massive cell death at a specific embryonic time period, in which survival of neurons is dependent on access to nerve growth factor (NGF) [34]. Motoneurons are another neuronal population that experience developmental apoptosis. In the developing rat, motoneurons are cleared between embryonic day 14 until postnatal day (p) 4 with half of the 6000 motoneurons undergoing cell death [35].

Much of the early work on developmental apoptosis used the PNS as a model system. Less was known about the importance of cell death in the central nervous system (CNS). The reason for the investigational lag is the two challenges that exist

with the study of the CNS. Brain regions are commonly larger than PNS ganglia, therefore apoptosis is much more difficult to detect. The other issue is the incomplete knowledge of how survival and death take place in the CNS. The neurotrophic theory, which will be discussed in greater depth later in the thesis, has been established in the PNS. Less is known on whether this model fits in the CNS as well.

Despite these considerations, groups have been able to investigate death in the CNS and have gathered important information regarding the process. The earliest CNS apoptosis event comes in the transition of the neural plate into the neural tube [36]. Later on ventral telencephalic and subplate neurons, which are important in the establishment of thalamocortical circuits, disappear via apoptosis [37]. Cell death has been further noted in numerous areas including the cortex [38], hippocampus [39] and midbrain [40].

In terms of temporal period, much of the apoptosis that occurs in the nervous system occurs over a wide range of developmental time, but at different frequencies. Apoptosis occurs in the embryo affecting the aforementioned subplate and ventral telencephalon neurons. Blaschke et al. narrow down programmed cell death to occur at its highest levels between embryonic days 14 through 18 in the developing mouse [41]. Apoptosis continues after birth, with peak numbers observed between p1 and p7 in mice [42]. By adulthood apoptotic events have decreased dramatically, which coincides with a decreased expression of proapoptotic proteins [43].

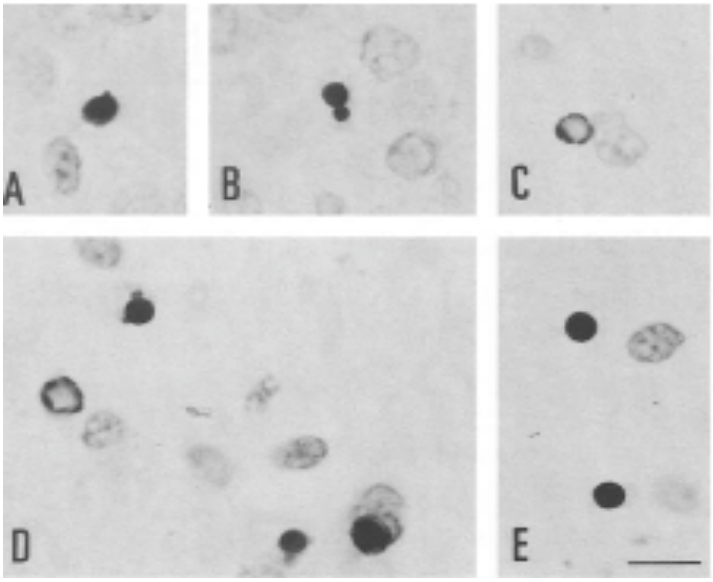


Figure 1-4. In-situ labeling of fragmented DNA, an indicator of cell death, in rat brain tissue. (A) p5 neocortex; (B) p3 subiculum; (C) p7 hilus of the dentate gyrus; (D) p5 subcortical white matter; (E) p7 subcortical white matter [38].

The areas of the nervous system and critical time periods affected by apoptosis have been discussed, but what about the apoptotic cells themselves? What specific cell types are removed during developmental apoptosis? Both postmitotic and proliferating neurons have been shown to undergo developmental apoptosis. Experiments by different groups have also revealed that glial cells die during development. The next paragraphs will introduce what is known about cell death in postmitotic, proliferating and glial cell populations.

One of the earliest findings in the study of programmed cell death was that a number of postmitotic neurons are affected by apoptosis. The neurons dying in the PNS are postmitotic, exhibiting mature neuronal characteristics such as the presence of axonal processes and the ability to synapse with their target tissue. In the developing

CNS, cortical areas comprised of postmigratory, postmitotic neurons are positive for terminal deoxynucleotidyltransferasedUTP nick end labeling (TUNEL), a method for marking apoptotic cells [38, 44].

Apoptosis can also clear cycling neural progenitors. Proliferating neuroepithelial cells and recently differentiated neuroblasts in the chick retina have been shown to undergo apoptosis [45]. The cell death that occurs can be described to happen in waves, with the highest incidences affecting these two neuronal populations. Progenitors in the SVZ and hippocampus, the two proliferative areas of the postnatal brain, have also been shown to undergo apoptosis [46, 47].

Given that glia make up the majority of cells in the nervous system, it is not surprising that they would also be targeted by developmental apoptosis. Unlike neurogenesis, which starts during embryonic development, most gliogenesis commences after birth in the rodent, lasting until the second postnatal week. The death of glia was widely studied in the context of the developmental death of oligodendrocytes in the rat optic nerve, where 10,000 cells die per day between p8 through p12 [48]. In the cerebellum, astrocytes are the main cell type to be cleared during postnatal development [49]. Similar to neuronal apoptosis, the death of glial cells is thought to be caused by limiting survival factors, and an overproduction of the glial cells themselves [48, 49].

1.2 Subventricular Zone

The development of the cerebral cortex is a rapid sequence of events that ends in the establishment of neural and glial populations within distinct brain structures. In

the embryonic brain, the ventricular zone (VZ) is a major site of neurogenesis. The VZ is a remnant of the neural plate and appears morphologically as a pseudostratified epithelium [50]. Radial glial cells (RGC) populate the VZ, and give rise to the neural progenitors in the embryonic brain. RGCs divide at the ventricular surface, producing a neuronal daughter cell that migrates, using the parent RGC as a scaffold, to the appropriate cortical layer. RGCs can also divide to self-renew, producing a clone [51]. The successive cycle of cell division and migration of neural progenitors results in the laminar structure of the cortex.

The landmark experiments of Pasko Rakic and of other research groups in the 1970s and 80's elucidated the formation of the cortical laminar structure. The resulting radial unit hypothesis states that the cortex is formed in an inside-out pattern, the most recently born neurons are situated in the most superficial layer of the cortex [52, 53]. This hypothesis also maintains that cortical neurons located in the same layer possess the same birth date. Following neurogenesis in the embryonic cortex and the birth of the organism, it was a widely accepted dogma that neural proliferation no longer occurred [54].

The validity of the idea that new neurons were no longer produced in the adult brain was challenged by the findings of Joseph Altman, then at the Massachusetts Institute of Technology. Altman lesioned the brains of adult rats and with the same needle injected (^{3}H)-thymidine, a precursor of chromosomal DNA that is incorporated into the nuclei of dividing cells. Glial cells close to the injury site incorporated the marker.

Surprisingly, neuroblasts in areas far from the injury site were also positive for the marker.

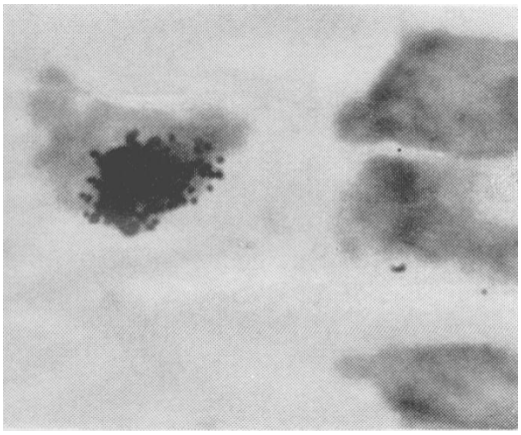


Fig 1-5. (^{3}H)-thymidine labeling of cortical neurons in adult Long Evans rats, 1 month after injection [55].

Altman's findings were met with skepticism by the neuroscience community and were largely ignored for two decades following the publication of his work. It took the experimental results from the laboratory of Fernando Nottebohm in the 1980's for acceptance of adult neurogenesis to gain momentum. Nottebohm's group found neurogenesis to occur in adult female canaries using (^{3}H)-thymidine labeling [56]. From this point on, a flurry of studies came out to support the occurrence of adult neurogenesis in a variety of animal species [57-59].

Since the work of Nottebohm's group a number of the events in adult SVZ neurogenesis have been described and published [60-62]. There is a wealth of information concerning the adult SVZ. There has been less focus, in comparison with the adult, in elucidating the character of this proliferative area in the neonate. The

perinatal SVZ is an important and distinct structure, differing from the adult in terms of cell types produced and sensitivity to apoptosis. This thesis will focus primarily on the neonatal SVZ neurogenic zone and the processes affecting it.

One might assume that postnatal neurogenesis as an exact recapitulation of embryonic neurogenesis. However, the areas of neonatal neurogenesis are more spatially restricted than their embryonic VZ counterpart. In the mammalian neonate, neurogenesis occurs in the cerebellum, SVZ, and the dentate gyrus of the hippocampus. Murine cerebellar neurogenesis goes on 2-3 weeks after birth, and produces a large pool of granule cell progenitors [63]. The SVZ and the hippocampus remain proliferative zones into adulthood.

Towards the end of embryogenesis and after birth, the postnatal SVZ expands in area while the VZ contracts significantly. The SVZ contains the largest pool of neural stem cells and lines the lateral ventricles (LV) in the neonate. The majority of the neuroblasts born in the SVZ travel towards the olfactory bulb (OB) via the rostral migratory stream (RMS). Once they arrive in the OB, the neuroblasts differentiate into periglomerular and granule olfactory interneurons or dopaminergic neurons, and integrate into the existing network of OB neurons [64, 65]. A detailed description of the events from the birth of the neuroblast to its arrival in the OB will be given.

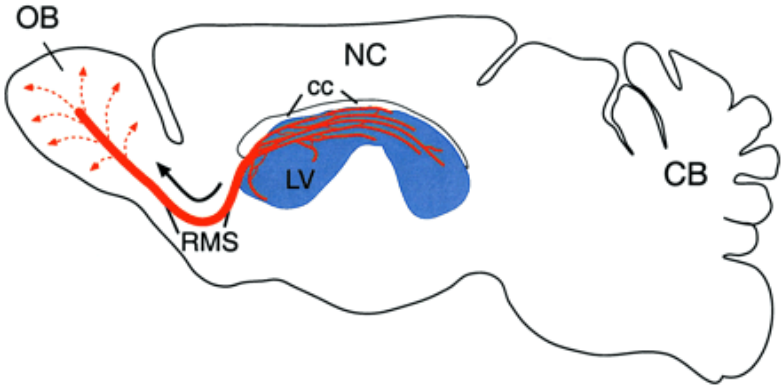


Fig 1-6. Migratory pathway of SVZ derived neuroblasts. Neocortex (NC), Corpus callosum (CC), Cerebellum (CB) [64].

The SVZ is a composite of distinct subregions and various cell types. The SVZ area can be subdivided into separate anatomical regions, the anterior SVZ, dorsolateral SVZ, lateral SVZ and septal SVZ [50]. The regions are divided based on their individual unique properties.

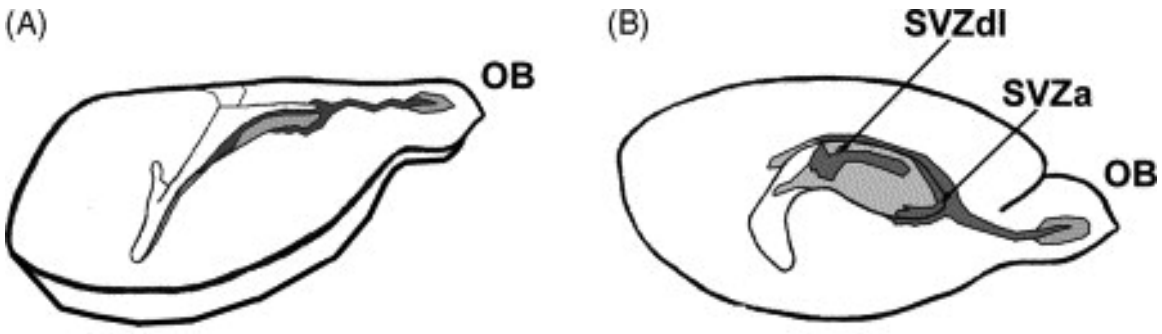


Fig 1-7. (A) Horizontal view and (B) sagittal view of the adult rodent SVZ, featuring the anterior (SVZa) and dorsolateral (SVZdl) subregions [50].

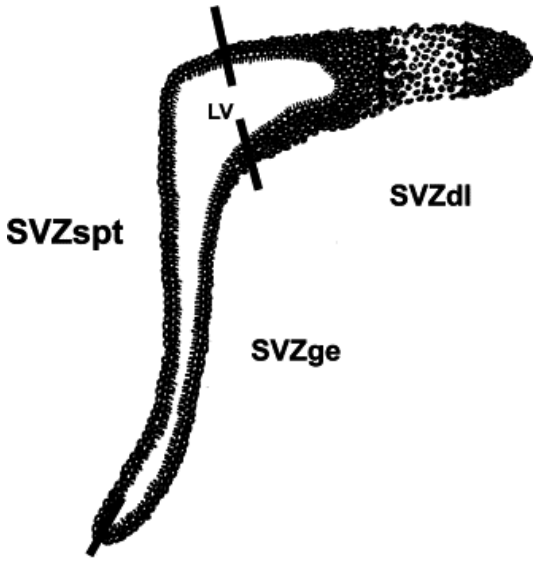


Fig 1-8. Coronal view of young rodent SVZ showing the septal SVZ (SVZspt), SVZdl, and lateral SVZ (SVZge) [50].

The anterior SVZ is located rostral to the frontal tip of the LV, and is the subregion where neuroblasts converge before they embark on their migratory journey towards the OB [50, 54]. The lateral SVZ is defined by the brain areas from which it originated, namely the medial ganglionic eminence and the lateral ganglionic eminence, and the septal SVZ is distinguished by its location next to the 3rd and 4th ventricles [50]. There is no consensus regarding a primary cell type generated from the septal or dorsal SVZ. A detailed characterization of these subregions will aid in forming a more accurate view of postnatal SVZ neurogenesis.

The SVZdl is discussed separately owing to the fact that it is a subregion of the SVZ that has been most studied, particularly in the neonate. Glial cells, both astrocytes and oligodendrocytes, are born in the perinatal SVZdl, which is located in the

dorsolateral aspect of the LV at the level of the anterior commissure [50, 66]. The SVZ cells can either be specified to the glial lineage while still in the SVZ or the cells can migrate into the cortex and differentiate into the glial lineage there [67]. The neonatal SVZdl demonstrates that the SVZ, at least during development, can produce cell types in addition to neuroblasts. It is worth investigating if other SVZ subregions have similar gliogenic potential as the SVZdl and whether cells in the other subregions are migratory as well.

SVZ gliogenesis was determined after the characterization of neurogenesis in the region. The presence of proliferating neural progenitors in the region throughout the postnatal life of the organism is what drew wide attention to the study of the SVZ. The other cell types residing in the SVZ interact with these progenitors, and amongst themselves, to give the SVZ niche its remarkable properties. The perinatal SVZ is comprised of 4 classes of cells, RGCs, mitotic and postmitotic neuroblasts, rapidly dividing transit amplifying cells (type C cells), and ependymal cells. In the neonate, RGCs persist from the embryo, and eventually give rise to astrocyte-like stem cells (type B cells) in the adult SVZ [68].

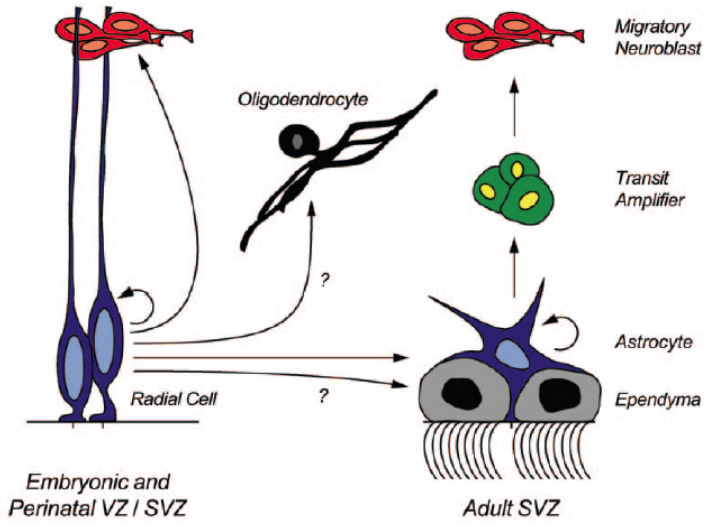


Fig 1-9. Cell types in the neonate and adult SVZ. Radial glia are the stem cells in the neonate SVZ, producing neural progenitors, glial cells, and ependyma. Neonate radial glial cells become type B cells in the adult SVZ. Type B cells divide into type C cells in addition to the cell types already mentioned [69].

Perinatal SVZ neurogenesis is similar to embryonic neurogenesis, in that it is the radial glial cells that produce the neural progenitors [69]. Radial glia undergo interkinetic migration to asymmetrically divide on the ventricular surface in the postnatal SVZ, producing neuroblasts [69]. It is still unclear whether radial glial cells asymmetrically divide to produce type B cells, mature and differentiate into the type B cells themselves, or both. By the end of the 2nd postnatal week, radial glial cells can barely be detected in the SVZ [69].

Neurogenesis is not the sole developmental process to occur in the postnatal SVZ. Apoptosis exists in parallel with proliferation, with cell death peaking in the first two weeks after birth and declining in adulthood [46]. Elimination of the proapoptotic protein, Bax, results in a significant decrease in cell death numbers in the p2 mouse SVZ [42].

The cell type protected from apoptosis in the Bax nulls was not identified. In the adult, when Bax is eliminated along with another proapoptotic protein, Bak, there is a gross accumulation of cells in the SVZ and hippocampus [70]. Similar double knockout experiments will need to be investigated in the neonate to determine the necessity of Bak, and other proapoptotic proteins in SVZ maintenance. These results from single and double knockout mice do, however, suggest that apoptosis is an intrinsic feature of the SVZ proliferative niche.

The SVZ neural precursors that survive migrate towards the RMS, the pathway to the OB [71]. Migrating neuroblasts in the neonatal RMS are not ensheathed by astrocytes until 3-4 weeks after birth [72]. Mitosis and apoptosis occur concurrently during neural precursor migration in the RMS, similar to what takes place in the SVZ [73]. It takes 26 days for BrdU labeled cells in the neonatal SVZ to reach their appropriate OB layer after injection [74]. Neuroblasts that arrive at the OB differentiate into olfactory interneurons in the periglomerular, granule cell, and external plexiform cell layers [75].

The final step in the journey of OB bound juvenile SVZ neuroblasts is their integration into the OB circuit. Neural precursors generated from the perinatal SVZ integrate preferentially into the external edge of the granule cell layer in the OB versus adult generated neuroblasts [74]. More neural precursors undergo apoptosis before they join the OB network. Apoptosis occurs in the postnatal OB much like the adult OB. However, cells in the neonatal OB possess longer survival times compared to their adult counterparts [74]. The majority of the surviving cells differentiate into GABAergic

granule neurons (95%) and the rest into GABAergic and dopaminergic periglomerular neurons (5%) [75]. Neuroblasts that survive and differentiate become indistinguishable from existing neurons in the OB [76].

In this introduction SVZ neurogenesis has been discussed from the birth of the neuroblast in the SVZ to its arrival in its final cortical destination. One of the ongoing questions in the field is the functional significance of continued neural proliferation in the brain. When it comes to SVZ neurogenesis, there is still no clear answer. Experiments looking at the effects of reducing or inhibiting postnatal neurogenesis have had conflicting results. The vast majority of experiments investigating the significance of SVZ neurogenesis have been performed in the adult, hence the results of these studies will be the ones discussed.

Some studies have shown the dependence of olfactory perceptual learning on neurogenesis. Infusion of AraC, a mitosis inhibitor, into the adult SVZ, significantly reduces the ability of mice to discriminate between two odors [77]. OB neurogenesis also appears to have a role in associative learning. Irradiation of the adult SVZ in mice reduced neurogenesis, and consequently led to decreased accuracy in recalling earlier odor memories [78].

However, there have been other experiments that make the argument against a role for SVZ neurogenesis in learning and memory. Adult mice, in which newly generated neurons are genetically ablated through a tamoxifen-inducible Cre recombinase expressed under the control of a nestin promoter, show no difference in their odor discrimination ability or odor-associated memory task performance compared

to controls [79]. It is possible that the difficulty of the task may be a determinant of whether or not new neurons are recruited [77]. More experiments in the adult will need to be performed to settle the conflicting roles of postnatal neurogenesis, with a special emphasis on determining the influence of task complexity on experimental results. Experiments with neonate animals will also need to be done to settle any similarities or differences in the role of SVZ neurogenesis on olfactory and learning behavior during development versus adulthood.

1.3 Neurotrophin Signaling and the Developing Brain

1.3.1 Ligands and Receptors

Neurotrophins (NT) are a family of secreted proteins that along with their complement receptors play essential roles in neuronal survival, differentiation, neural precursor proliferation, and many more essential processes [80]. NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) constitute the mammalian NT family. Each ligand binds to a specific tyrosine kinase receptor; NGF binds preferentially to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC. The actions of NTs and their cognate receptors extend beyond the nervous system, with research showing roles in heart development and immune function [81].

Before discussing the properties and functions of each individual NT, it is worth recognizing this family's shared characteristics. For all NTs to become functionally active, they must be cleaved into a "mature" form, a 12kDa, stable non-covalent dimer [82]. The endoprotease furin and proconvertases are responsible for this cleavage. The

pro form is cleaved at highly conserved dibasic amino acid cleavage sites within the *trans* Golgi network and endoplasmic reticulum [82, 83].

The potential biological activity of the uncleaved NT “pro” form became a widely studied question in the field of NT research, given that it was only the mature form that interacted with Trk receptors. What was the biological significance of the pro form? The answer lies with the p75 receptor. The NT pro form preferentially binds to the p75 receptor to activate cell signaling cascades that end in cellular activities such as apoptosis. This finding highlights the delicate balance and interplay between Trk and p75 receptor signaling, with Trk signaling promoting survival and p75 signaling favoring apoptosis [83, 84].

NTs share a common repertoire of signaling events once their complement Trk or p75 receptor is activated. All the activated Trks are phosphorylated at 10 evolutionary conserved tyrosine residues within the cytoplasmic domain of the receptor [85]. In brief, once a Trk receptor is activated it can initiate the Ras/ERK protein kinase pathway, the phosphatidylinositol-3 kinase/Akt kinase pathway and the phospholipase C-λ1 pathway [85]. The effects of the receptor activation vary depending on which signaling pathway is recruited.

It is worth noting that Trks come in multiple isoforms. The described signaling events that follow Trk receptor activation are associated with activation of the full-length receptor. Only TrkB and TrkC receptors have existing truncated forms, which are non-catalytic. The two truncated forms of TrkB are designated T1 and T2, with both lacking their cytoplasmic tyrosine kinase domains [86]. TrkC has more isoforms than TrkB,

numbering at least 8 [87]. In addition to the full and truncated forms, there are TrkC isoforms that contain inserts in the kinase domain [88]. It has been proposed that truncated Trk receptors function as negative regulators by sequestering NTs and keeping them from the full-length, active receptors [86, 87].

The other class of NT receptor is p75. Once p75 is activated, there is an increase in the levels of jun N-terminal kinase (JNK), NF-κB and ceramide, as well as modulation of Rho activity [82]. Looking at the list of proteins affected by p75 makes sense given that p75 is a member of the superfamily of death receptors that counts tumor necrosis factor and Fas receptors as members. The actions of JNK, in particular, are relevant due its involvement in the activation of proapoptotic proteins Bim and PUMA. Activation of Bim and PUMA is just one of the ways in which p75 can cause apoptosis.

p75 can be viewed as a schizophrenic receptor, with the effects of its signaling dependent on the cellular context. The proapoptotic function of p75 is correlated with the absence of Trk receptors on the same cell [89]. In contrast, if p75 is present with Trk receptors, then there is increased binding affinity of mature NTs to their complement Trk receptor [90, 91]. The direct association of the Trks with p75 increases binding efficacy in different ways. Two potential mechanisms have emerged in the field.

The first proposed mechanism is that p75 binds a NT, and concentrates it or presents it to its Trk receptor in the most compatible binding conformation [92] This has been shown in experiments in which the effects of p75 on Trk binding efficacy are lost when p75 is prevented from binding to NGF [93]. The contrasting view is that p75 activation is not necessary for its effects on Trks. p75 provides an allosteric effect that

does not necessitate its signaling via NT binding [92]. Future experiments will look further into the importance of p75 signaling on Trk binding optimization.

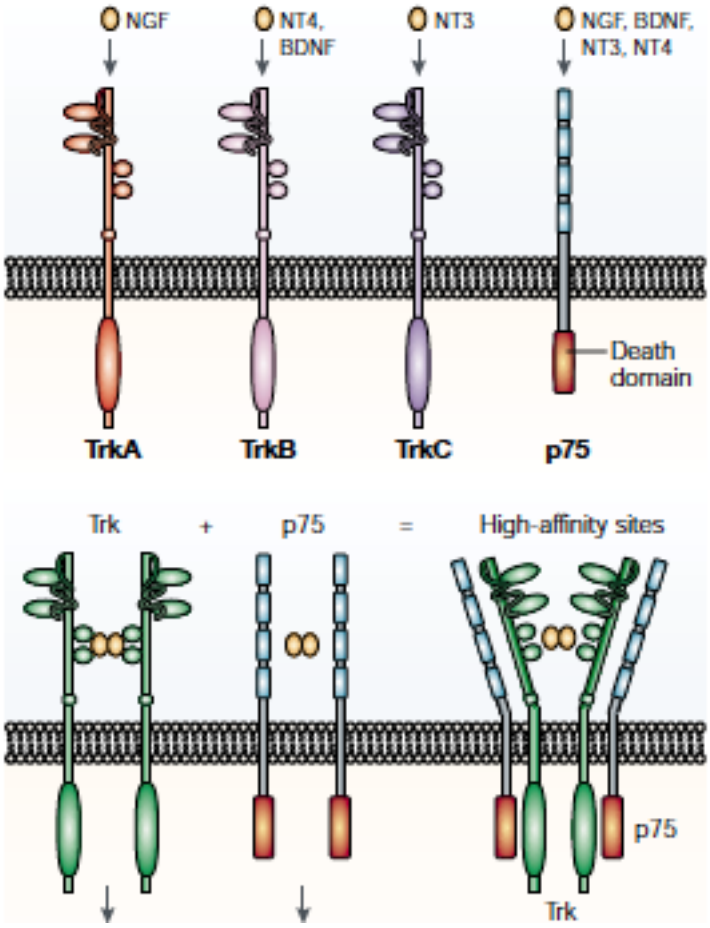


Figure 1-10. NTs and their preferred receptor binding partner. The direct association between Trks and p75, which give rise to higher affinity ligand receptor binding sites, is also shown [82]

Before receptor activation, and the intricate signaling events that follow, there exists the secreted NT. The NTs differ in their spatial expression patterns and influence a wide and varied range of activities in the nervous system. A study of each individual NT reveals how they come to modulate diverse processes from proliferation to synapse formation.

(NGF) NGF was the first NT to be extensively studied. The developmental biologists Viktor Hamburger and Rita Levi-Montalcini performed experiments on chick limb bud removal that resulted in the death of motor nerve cells that normally innervate the bud [94]. The search for the mechanism underlying the observed motor neuron apoptosis ultimately led to the identification of NGF [94].

The role of NGF as a survival factor was supported by studies in which NGF was injected into chick embryos, and led to an increased density of sensory and sympathetic ganglia [94]. NGF is now broadly recognized as an essential component in the survival of neurons in the PNS, particularly that of sympathetic and sensory neurons [95]. In addition to its prosurvival role, NGF signaling has been shown to cause cellular differentiation. Experiments that expose the PC12 cell line to NGF, show the cells sprouting neurites and developing other mature neuronal features [96]. In both cases, NGF causes the phosphorylation of TrkA, and the activation of one of the mentioned signaling cascades (Ras/ERK protein kinase pathway, phosphatidylinositol-3 kinase/Akt kinase pathway and phospholipase C-λ1 pathway).

(BDNF) In 1982 it was published that a new member of the NT family, BDNF, had been isolated and characterized from the pig brain [97]. Before the identification of BDNF, there had been a growing consensus that NT signaling might only be functionally relevant in the PNS. However, research has shown BDNF signaling to have a plethora of effects in the nervous system, particularly in the CNS. TrkB, the BDNF receptor, is the most abundant Trk receptor in brain tissue. BDNF knockout mice die within 48 hours

after birth (a minority survive for up to 2 weeks) and have significant motor abnormalities [98]

Studies on the effects of BDNF elimination, coupled with BDNF rescue experiments, have highlighted the important developmental processes that require BDNF. The survival of virtually all primary sensory neurons depends on the presence of BDNF [98]. Cerebellar neurons are also affected by the loss of BDNF. Mice that lack BDNF display abnormal dendritic arborization of Purkinje neurons, increased granule neuron cell death, and disrupted cerebellar layer formation [99]. Additional studies have shown a role for BDNF beyond survival, particularly in synapse formation and plasticity [100, 101].

Despite these deficits, the phenotype of BDNF nulls is much less severe compared to TrkB nulls. There is no gross anatomical difference in the hippocampus, cortex and retina between BDNF knockouts and wild type [99]. This finding could be largely due to compensation by NT-4, which would allow TrkB signaling to continue in the BDNF knockouts.

(NT-3 and NT-4) NT-3 and NT-4 preferentially bind to TrkC and TrkB receptors, respectively. NT-3 and TrkC are expressed at high levels in the cortex, cerebellum, and in the dentate gyrus of the hippocampus [102]. Temporally, both NT-3 and TrkC mRNA peak between p1 to p14 [103]. Expression of NT-3, but not TrkC, declines significantly in adulthood. The continued temporal presence of NT-3 makes a case for it's specific involvement in the developing brain.

NT-3 has been shown to exert effects on various neuronal populations. NT-3 promotes fiber outgrowth by sympathetic ganglia, and can enhance the proliferation of hippocampal precursors [104]. NT-3 is also the only NT that has been observed to influence the development and function of the enteric nervous system [105]. NT-3 knockouts have provided additional information on the groups of neurons and processes that depend on this NT. Most NT-3 knockouts die within 48 hours, have a massive loss of propioceptive sensory neurons and display a decreased spinal cord diameter [106]. NT-3 signaling has also been shown to not be limited to neuronal cells. There is a reduction in oligodendrocytes and astrocytes in NT-3 and TrkC knockouts [107].

NT-4 shares binding affinity to TrkB along with BDNF. One of the most salient questions is what differentiates NT-4 binding to TrkB. NT-4, unlike BDNF, is expressed primarily outside the brain. NT-4 null mice are able to survive in contrast with TrkB and BDNF knockouts that mostly die close to birth [108]. The survival of the NT-4 nulls argues for the ability of BDNF to compensate for the lack of NT-4 in certain cell populations. Other roles for NT-4 are in promoting the survival of D-hair afferents in the dorsal root ganglion, differentiation of hippocampal neuronal precursors (along with BDNF and NT-3), and formation of ocular dominance columns [109-111].

1.3.2 Roles in Survival: The Neurotrophic Theory

The existence of NTs has become inextricably linked with the neurotrophic theory. The neurotrophic theory, born from the early experiments of Hamburger and Levi-Montalcini, postulates that populations of neurons, particularly during development, are in constant competition for a limited supply of NTs produced by their synaptic target. A

neuron that is unable to access an adequate amount of their appropriate NT is cleared from the tissue via apoptosis. The NTs therefore act as a survival factors, and have been shown to be critical to both proliferative neuroprogenitors and postmitotic neurons. The survival role of NTs has even been further extended to include glial cells [112].

Cell death of numerous neuronal populations can be explained by the neurotrophic theory. Sensory and sympathetic neurons in the PNS have been the most extensively studied group, with their survival depending on access to NGF. It has been estimated that about 50% of these neurons die via apoptosis during development. NGF treatment of neonatal animals rescues these sensory and sympathetic neurons from cell death, while administration of NGF neutralizing antibody results in the death of almost all these neurons [48].

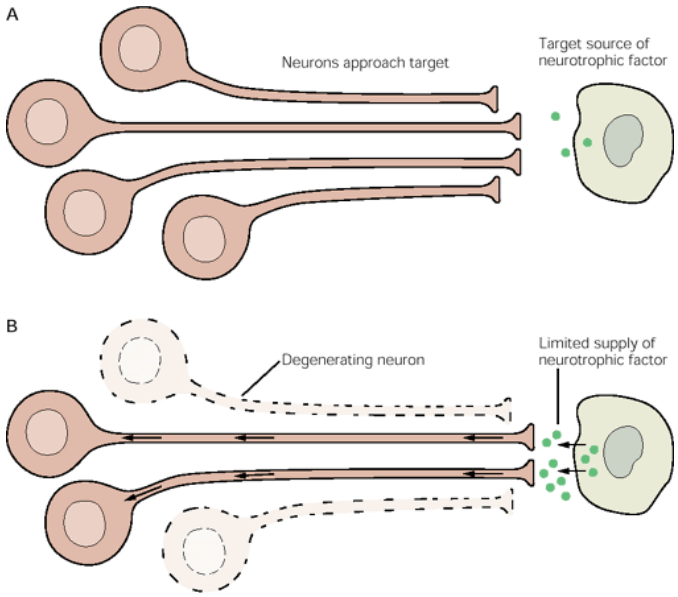


Figure 1-11. The neurotrophic theory describes developmental apoptosis to be dependent on the availability of NTs. Neurons that are unable to access their required NTs from their target undergo cell death [113].

Despite the wealth of examples of the neurotrophic theory at work in the nervous system, particularly outside the brain, there are still notable apoptotic incidences that the theory cannot adequately explain. Southwell and colleagues explored the developmental cell death of cortical inhibitory neurons [114]. They found no significant difference in the amount of dying cells when various numbers of inhibitory neurons were transplanted into a host neocortex [79]. This is surprising given the knowledge that there is a limited supply of NT to support developing neurons. There was also no significant difference in cell death with the transplantation of WT or TrkB null neurons into the host [79].

The death of neonatal lateral SVZ cells as a consequence of BDNF elimination, also serves as another example of the limitations of the neurotrophic theory in explaining all CNS developmental apoptosis. In the study, a premigratory population of cells in the ganglionic eminence zone of the neonatal mouse SVZ, a population that has yet to synapse to a target, undergoes apoptosis when BDNF or TrkB is removed [40, 115]. The neurotrophic theory would not have predicted the dependence of this cell population on NT support, given that these cells have not synapsed to a NT-producing target source. The other remaining SVZ subregions have yet to be studied to see whether this neurotrophic dependence is a generalized phenomenon or specific only to the lateral SVZ.

The results of the studies mentioned casts doubt on the universality of the neurotrophic theory, particularly the exact mechanism underlying how developing cell populations can encounter NTs for survival. TrkA, unlike other Trk receptors, acts as a

dependence receptor in the PNS and explains why the removal of TrkA results in a more dramatic loss of neurons compared to the elimination of other Trks in the CNS [80]. Remaining questions regarding the aspects of premigratory neuronal population dependence on NT, the potential source of the NT for these cells, and the possibility of interaction between NT signaling and other survival cues in the developing CNS, such as growth factors and neurotransmitter release, necessitates further investigation of NT in development.

1.4 Rationale and Outline

This thesis is an in-depth investigation of the features and mechanisms of apoptosis in the developing SVZ. Apoptosis, as already described in the previous sections, is an essential feature of nervous system development and is necessary for normal CNS and PNS anatomical development and function. Apoptosis has been investigated in the cortex and other CNS regions, but information on its occurrence in the neonatal SVZ is lacking and incomplete. In addition, most studies in the field have focused on describing the adult SVZ niche, with little emphasis on the events that take place in the neonate. This thesis will serve to enrich what is known about cell death in this region during this time, and allow for comparisons of apoptotic properties between the neonate and adult SVZ.

Chapter 2 will discuss the materials and methods employed in the study of apoptosis in the neonatal SVZ. Chapter 3 will summarize and discuss the results collected from experiments investigating the relative levels of apoptosis in the SVZ subregions during the first week of neonatal development in rodents and the cell types undergoing cell

death. A description of the migratory path of cells residing in the medial SVZ, an area in which extensive death of post-mitotic neurons occurs just after birth will also be presented. Chapters 4 and 5 are dedicated to covering the mechanisms governing cell death in the developing SVZ, on both an intracellular and extracellular level. Finally the last chapter, chapter 6, will focus on the conclusions of this thesis and implications from the experimental data that can add towards the development of therapeutic strategies most commonly associated with SVZ research, such as cell transplant therapy.

Chapter 2. Materials and Methods

2.1 Animals

All rodent procedures were approved by the Columbia University Institutional Animal Care and Use Committee. Experiments were performed on newborn (p0&p1) and young (p7) Sprague Dawley rats (Charles River, Wilmington, MA). Bim and PUMA wild type and knockout mice (p0 & p7) used were generated and provided by Dr. Richard Libby and colleagues [116, 117].

2.2 Antibodies and Peptides

See Table 2-1 for a complete list of antibodies used.

Table 2-1.

Antigen	Species	Dilution	Source	Catalog #
Bim	Rb polyconal	1:100	Cell Signaling	2819
Bim peptide	Rb	1:50	Cell Signaling	1910B
BLBP	Rb polyclonal	1:200	Millipore	ABN14
Cleaved Caspase 3	Rb polyclonal	1:100	Cell Signaling	9661
HuD	Rb polyclonal	1:100	Millipore	AB5971
Ki67	Ms monoclonal	1:100	Vector Labs	VP-K452
Mcm2	G polyclonal	1:100	Santa cruz	sc-9839
Neuro D	G polyclonal	1:50	Santa cruz	sc-1084
p75	Ms monoclonal	1:1000	Millipore	MAB365
PDGFRα	G polyclonal	1:40	R&D Systems	AF1062
pH2ax	Ms monoclonal	1:200	Abcam	ab26350

pH2ax	Rb monoclonal	1:200	Cell Signaling	9718
pTrkB	Rb polyclonal	1:100	Abcam	ab75173
S100β	Ms monoclonal	1:100	Millipore	MAB079-1
TrkB	Rb monoclonal	1:100	Cell Signaling	4607

Ms, mouse; Rb, rabbit; G, goat

2.3 Immunohistochemistry

Rats were anaesthetized and then perfused with 4% paraformaldehyde (PFA). The brains were dissected and fixed overnight in 4% PFA at 4^{o}C. Following fixation, brains were transferred to 30% sucrose solution at 4^{o}C. Brains were serially cut at 20μm using freezing microtome.

All tissue sections were submerged in 1x PBS + 0.1% Triton for 10 minutes before being placed in a rice cooker for antigen retrieval (10mM citric acid; pH 6.0).After allowing to cool down, slides were incubated for 1 hour with SuperBlock® 1x PBS (Pierce) + 0.1% TritonX-100 solution at room temperature (RT). After blocking, slides were exposed to primary antibody at 4^{o}C overnight. Sections were washed 3 times with 1xPBS + 0.1% Triton and followed by addition of species appropriate Alexa Fluor-conjugated secondary antibody (Invitrogen) for 1 hour at RT. Slides were washed 3 times with 1x PBS + 0.1% Triton, and mounted with DAPI containing mounting media.

All staining except for pH2ax was performed using a biotinylated secondary antibody. Blocking solution for endogenous Avidin/Biotin binding (Vector Labs cat#SP-2001) was added to the SuperBlock® + 0.1% Triton and primary antibody solution for these sections. Slides were then exposed to species appropriate biotinylated secondary

antibody (Vector Labs) and followed by treatment with Fluorescein Avidin D (Vector Labs) for 1 hour at RT.

2.4 Stereotactic Injection

Newborn (p0) rats were anaesthetized, and a 1.0-1.5 cm incision was made in the midline of the scalp in order to locate the bregma. Animals were positioned in the head mold and injected with 2 μl of GFP tagged empty vector lentivirus (LV-GFP) K252A (Calbiochem, Billerica, MA; 50 μg/ml), TrkB IgG (R&D Systems, Minneapolis, MN; 50 μg/ml), and Goat IgG (R&D Systems, Minneapolis, MN; 50 μg/ml), in separate experiments. Injection coordinates used were 1mm anterior and .25mm lateral to bregma, and at a depth of 2 mm from the cortical surface. Injections were done with a 50 μl syringe (7637-01, Hamilton Company, Reno, NV) at an injection rate of 0.2 μl/ml. Following injections, pups were returned to their mothers and then sacrificed one day later at p1 or seven days later at p7.

2.5 Microscopy and Cell Quantification

Double labeled immunohistochemistry images were obtained with a hybrid spinning disc confocal microscope system (PerkinElmer) using an ORCA-ER camera (Hamamatsu) at 40x objective power. Images were analyzed using Volocity software suite. The remaining immunohistochemical images were obtained using Axiophot camera and Zeiss Axioplan 2 microscope, and imaged with Axiovision software. ImageJ software was used to measure the SVZ subregion areas. Apoptosis quantification was performed by dividing the total number of apoptotic cells by the total area of the SVZ subregion.

2.6 Statistical Analysis

All statistical analyses were performed using Graphpad Prism 5 for Mac software (Graphpad, La Jolla, CA).

Chapter 3. Characterization of Apoptosis in the Developing SVZ

3.1 Introduction

Apoptosis in the developing rodent neocortex has been reported to occur at its peak levels in the SVZ, between p0 and p14 [46, 118]. The incidence of cell death declines in this area, and in much of the brain, during the maturation of the neonate into the adult. From the initial observations and description of apoptosis in the neonatal SVZ, little progress has been made in fully characterizing this event. The biological significance, as well as the mechanisms that guide SVZ cell death during this time period remains largely unanswered.

A pressing question in the field relates to the amount of cell death occurring and the identity of the cells cleared during this time of SVZ developmental apoptosis. Although SVZ apoptosis has been quantified previously, the subregions in which they occur had not been taken into account. Why is it worth noting the SVZ subregions when quantifying apoptotic cells? The reason lies in the inherent differences between the SVZ subregions in terms of their embryonic origin and what cell type each region produces (see Chapter 1.2). A majority of the SVZ subregions, including the medial SVZ, still await further detailed characterization. Based on this information, the SVZ, at least during development, cannot be taken as a uniform whole.

Another existing question about the SVZ concerns the cell types removed via apoptosis. Earlier studies did not look at specific markers expressed by dying cells at the beginning of postnatal development [46, 119]. What has been reported is that between p8 and p14, there is a change in the composition of the apoptotic population.

At p8 the primary apoptotic cells that were able to be identified were GFAP+ positive astrocytes [118]. However by p14, it is GST-π+ oligodendrocytes that comprise the majority of dying cells [118]. Despite the observation of astrocytes and oligodendrocytes undergoing apoptosis during this time period, the vast majority of apoptotic cells did not express any of the cell specific markers used.

Finally, much of the work investigating apoptosis in the SVZ has employed TUNEL, in-situ end labeling (ISEL) or cleaved caspase 3 immunostaining. TUNEL and ISEL both detect apoptotic cells but are time intensive, and in the case of TUNEL, many tissue sections are destroyed through Proteinase K treatment. Cleaved caspase 3 is another widely used marker for apoptosis. Immunostaining for the protein is straightforward, with tissue sections remaining intact. The problem with solely using cleaved caspase 3 as an apoptosis marker is the fact that caspases are activated in cellular events other than apoptosis. Caspase 3 has been shown to have roles outside of apoptosis, particularly in synaptic plasticity and neurite pruning [120]. Therefore, it is difficult to delineate the apoptotic role of cleaved caspase 3 without the use of another antibody marker. Employment of an additional marker would strengthen the accuracy of identifying cell death events in tissue.

Throughout this chapter the marker phosphorylated histoneH2ax (pH2ax) will be used in combination with cleaved caspase 3 to mark dying cells. When a cell is presented with a sufficient stress stimulus the mitochondrial membrane is compromised, leading to the release of apoptosis inducing factor and endonuclease G. Once in the cytoplasm, both apoptosis inducing factor and endonuclease G translocate into the

nucleus, and promote DNA strand breaks. HistoneH2ax is phosphorylated upon DNA breakage. Studies have demonstrated the coincidence of pH2ax expression and cellular apoptosis [121, 122]. The following thesis chapter will verify the usefulness of pH2ax as a robust marker of apoptotic cells, along with presenting the amount and cell types affected by SVZ apoptosis and the migration pattern of p0 medial SVZ cells.

3.2 Results

3.2.1 Immunohistochemical Measures of Apoptosis

In order to verify that pH2ax was accurately marking cells undergoing apoptosis, double immunostaining was performed with a cleaved caspase 3 antibody on p0 rat frozen coronal brain sections (Fig. 3-1). Double immunostaining for pH2ax and cleaved caspase 3 showed that many SVZ cells expressing one death marker, also expressed the other marker. A majority of the cleaved caspase 3+ cells (77%) stained positive for pH2ax (Fig. 3-1, Student's t-test, $t=9.835$, $p < 0.005$). The same was true for pH2ax+ cells, 80% of cells positive for this marker also expressed cleaved caspase 3 (Fig. 3-1, Student's t test, $t=12.69$, $p < 0.005$). There were also groups of cells that expressed one apoptotic marker alone. This could be explained by cleaved caspase 3 and pH2ax being active at different time points in the intrinsic apoptosis pathway [121]. It is possible that cleaved caspase 3 or pH2ax has yet to be expressed in the cells that stained for pH2ax or cleaved caspase 3 alone. These results indicate that pH2ax marks much of the same dying cell population as cleaved caspase 3, and that both identify a similar proportion of apoptotic cells in the population. Thus, pH2ax can be used as effectively as cleaved caspase 3 to identify apoptotic cells in tissue.

Figure 3-1.

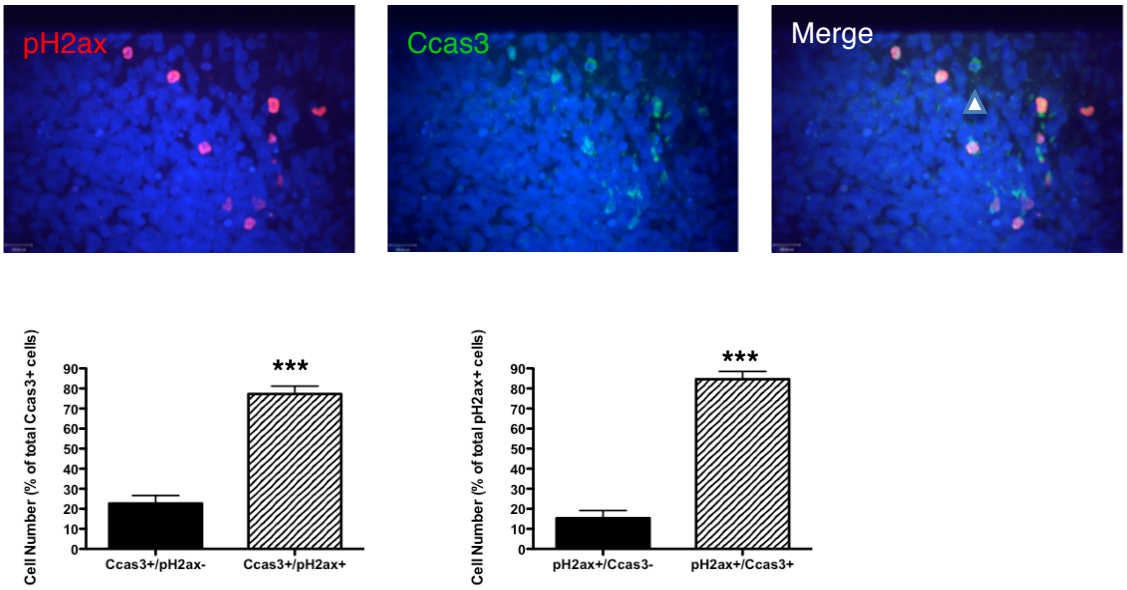


Figure 3-1. pH2ax and cleaved caspase 3 mark similar populations of dying cells. Immunohistochemistry of pH2ax and cleaved caspase 3 in the p0 medial SVZ of frozen rat coronal sections. pH2ax and cleaved caspase 3 individual confocal images are shown, along with stacked images for both markers. The arrow denotes a single stained cell for cleaved caspase 3 alone, with the remainder of the cells being double stained with both markers. The proportion of cells coexpressing both markers is presented as a proportion of total cleaved caspase 3 + cells (n=3 brains) and total pH2ax + cells (n=3 brains). Topro3 is shown in blue, pH2ax is shown in red, and cleaved caspase 3 is shown in green. All error bars represent s.e.m. Scale bar, 100 μm. ***$p < 0.005$. Ccas3, cleaved caspase 3.

3.2.2 Incidence of Apoptosis in Postnatal SVZ Subregions

3.2.2.1 p0 SVZ

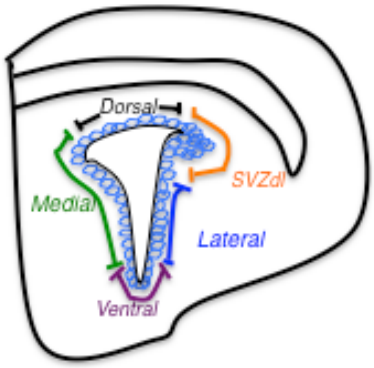
Given the confirmed utility of pH2ax for identifying apoptotic cells, immunohistochemistry with pH2ax antibody was used to quantify dying cells in the p0 SVZ. The selection of p0 as an SVZ time point to investigate is based on the literature description of SVZ apoptosis occurring at its highest levels in the first postnatal week [46].

The p0 SVZ was divided into 5 different subregions, the dorsolateral (SVZdl), lateral, ventral, medial and dorsal SVZ, and apoptotic cells were quantified for each subregion (Fig. 3-2C). Six coronal brain sections per animal were assessed, and the density of apoptotic cells was calculated by dividing the total number of pH2ax+ cells in each subregion by the total subregion area.

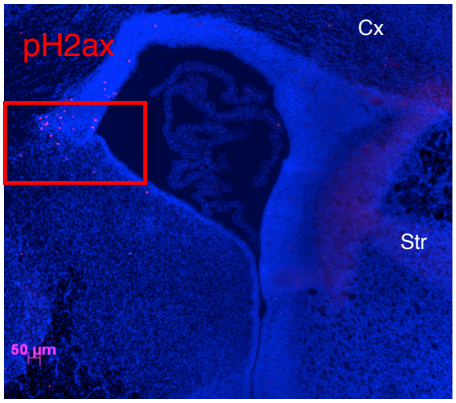
Apoptosis was detected in the p0 neonatal SVZ (Fig. 3-2B, Fig. 3-2C). The medial SVZ contains a significantly greater density of dying cells compared to the other subregions (Fig. 3-2C, analysis of variance (ANOVA), $F=17.47$, $p < 0.0005$). The dorsal SVZ has the second highest density of dying cells after the medial SVZ, but the dorsal SVZ apoptotic cell density was not significantly higher than the lateral, dorsal and ventral SVZ subregions. These results indicate the apoptosis does not occur uniformly in the p0 SVZ; rather there is a preference for apoptotic events to occur in the medial subregion.

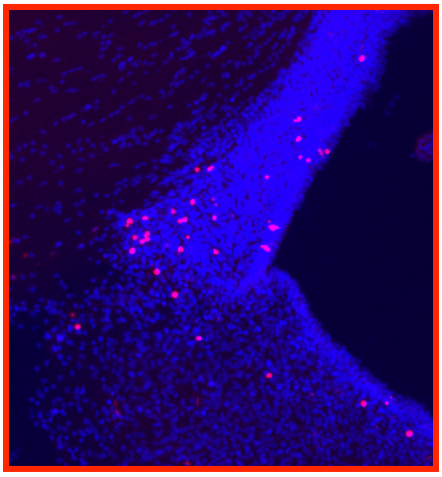
Figure 3-2.

3-2A



3-2B





3-2C

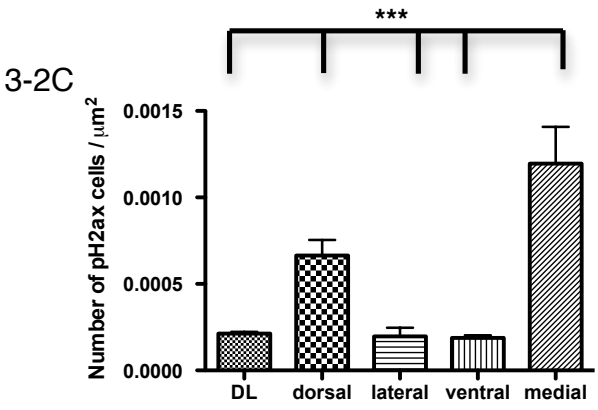


Fig 3-2. The medial SVZ has the highest density of apoptotic cells amongst the SVZ subregions at p0. Immunohistochemistry for pH2ax on rat frozen coronal sections **(A)** SVZ diagram highlighting the subregion boundaries **(B)** Immunohistochemistry for pH2ax in the p0 SVZ (red). DAPI staining is shown in blue. Red inset box shows location of higher magnification panel on the right **(C)** Quantification of total pH2ax+ cells per subregion area in the p0 SVZ (n=3 brains). Error bars represent s.e.m. Scale bar, 50 μm. ***p < 0.005. Str, striatum; Cx, cortex.

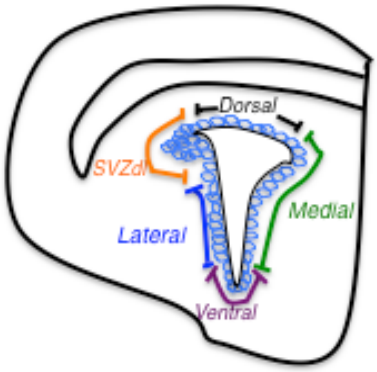
3.2.2.2 p7 SVZ

Looking at two different developmental time points allows for the determination of whether SVZ apoptosis undergoes any significant quantitative changes during the neonatal temporal window. Therefore the same method for detecting and quantifying apoptosis in the p0 SVZ was employed in the p7 SVZ.

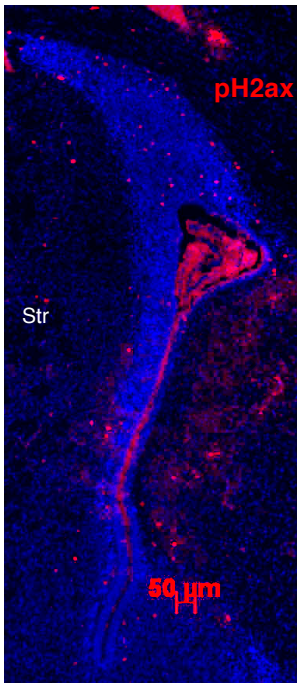
Apoptosis was detected in the p7 SVZ, but with no noticeable clustering of pH2ax+ cells in the medial subregion as there was in the p0 SVZ (Fig. 3-3B). pH2ax quantification revealed the significant differences in relative density of cell death among the various subregions was no longer detectable. There was no statistical difference in apoptotic cell densities amongst the different SVZ subregions at p7 (Fig. 3-3C, ANOVA, $F=2.674$, $p = 0.0944$). These experiments indicate that the frequency of apoptotic events in the SVZ subregions changes over developmental time, between p0 and p7.

Figure 3-3.

3-3A



3-3B



3-3C

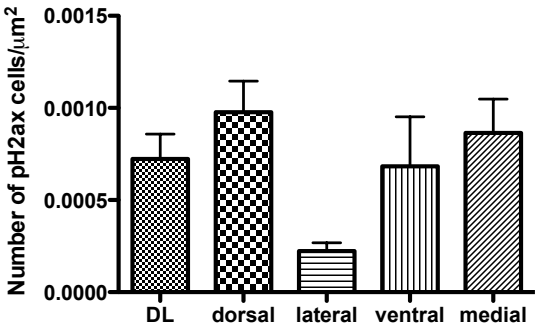


Fig 3-3. There is no significant difference in the density of apoptotic cells amongst the SVZ subregions at p7. Immunohistochemistry for pH2ax on rat frozen coronal sections **(A)** SVZ diagram highlighting the subregion boundaries **(B)** Immunohistochemistry for pH2ax in the p7 SVZ (red). DAPI staining is shown in blue **(C)** Quantification of total pH2ax+ cells per subregion area in the p7 SVZ (n=3 brains). Error bars represent s.e.m. Scale bar, 50 µm. ***p < .0005. Str, striatum.

3.2.3 SVZ Cell Types Undergoing Apoptosis

3.2.3.1 p0 medial SVZ

It was important to characterize the cell types being cleared by apoptosis at p0. To identify dying cells, double immunofluorescence was performed with either cleaved caspase 3 or pH2ax as an apoptosis marker along with various neonatal SVZ cell type specific markers (Fig. 3-4). The medial SVZ in particular was investigated at p0, given that it is the area with the highest density of apoptotic cells at this age (Fig. 3-4A, Fig. 3-4B). Because the SVZ is a proliferative area, the cells were first assessed for their mitotic potential. There was abundant staining for ki67 in the p0 medial SVZ, yet none of the dying cells were positive for this proliferation marker (Fig. 3-4A, Fig. 3-4B). This result was confirmed by the observed lack of colocalization of apoptotic cells with another proliferative marker minichromosome maintenance protein Mcm2 (data not shown).

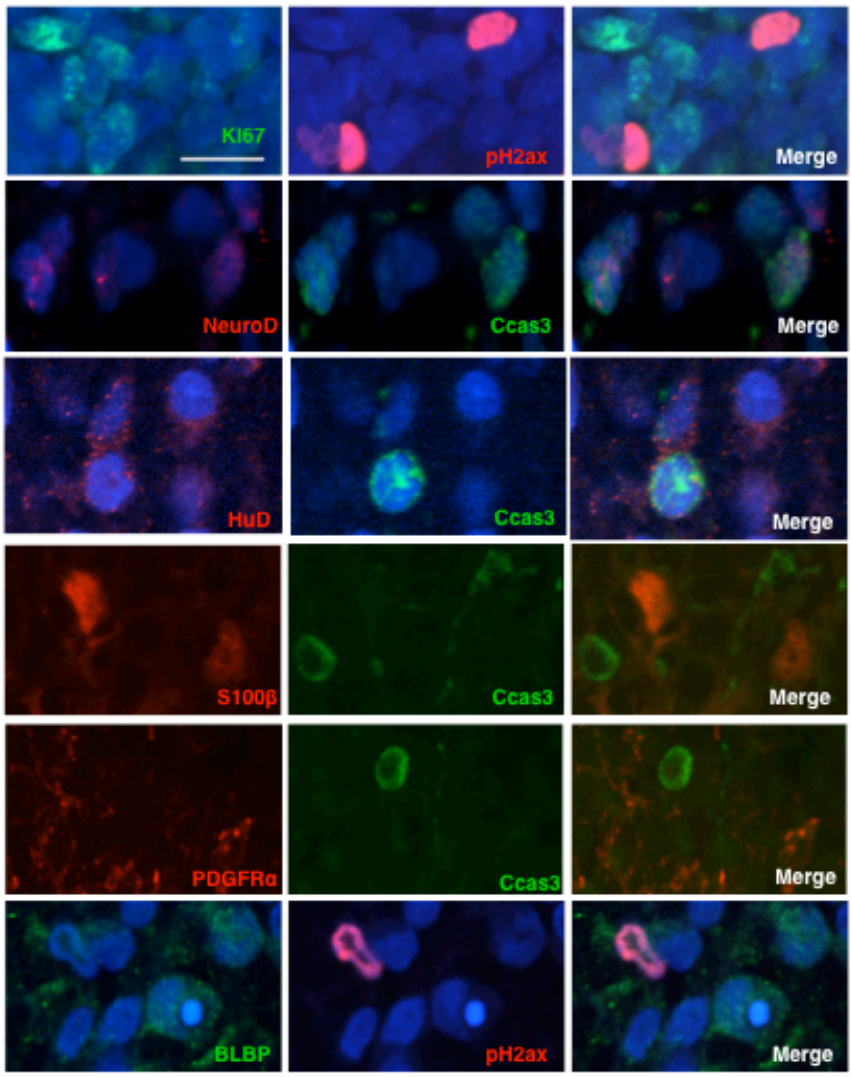
Apoptotic cells were next evaluated for postmitotic cell markers. In terms of specific cell types, most of the apoptotic cells expressed the BHLH transcription factor, NeuroD, which marks immature neurons (69%, Fig. 3-4A, Fig 3-4B). Immunostaining with another immature neuronal marker, HuD, also gave a similar proportion of labeled apoptotic cells (Fig. 3-4A).

A small number of the dying cells were positive for the astrocyte marker S100β (15%, Fig. 3-4A, Fig. 3-4B), or the early oligodendrocyte marker PDGFRα (4%, Fig. 3-4A, Fig. 3-4B). Many cells in the p0 medial SVZ were positive for the radial glial marker, brain lipid binding protein (BLBP), but none of the apoptotic cells expressed it (Fig. 3-4A,

Fig. 3-4B). Taken together, these experiments show that postmitotic immature neurons comprise the majority of dying cells in the p0 medial SVZ. A smaller portion were non-dividing astrocytes or early oligodendrocytes, and about 10% were not identified.

Figure 3-4.

3-4A



3-3B

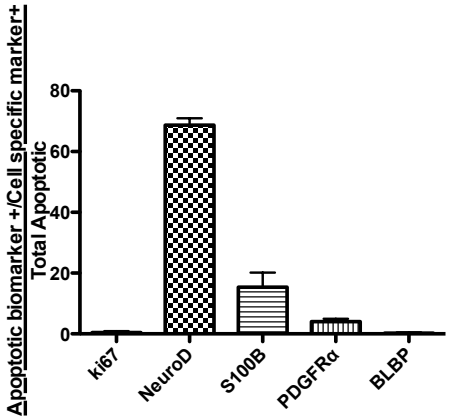


Fig 3-4: Postmitotic immature neurons comprise the majority of apoptotic cells in the p0 medial SVZ. **(A)** Confocal images of p0 medial SVZ co-stained for SVZ cell markers and pH2ax or cleaved caspase 3. Topro3 staining is shown in blue. **(B)** Fraction of p0 medial SVZ apoptotic cells expressing each of the different cell type markers shown in 3-4A. The majority of apoptotic cells express NeuroD, an immature neuronal marker. Six coronal sections per brain were assessed for each cell marker used (n=3 brains). Error bars represent s.e.m. Scale bar, 43 μm. Ccas3, cleaved caspase 3.

3.2.3.2 p7 SVZdl

To find out whether the same cell types were eliminated later in development, and in a different subregion of the SVZ, the p7 SVZ was assessed with the same apoptotic and cell markers used in the p0 medial SVZ (Fig. 3-5A, Fig. 3-5B). Because the p7 SVZ failed to show any significant differences in apoptosis density amongst the subregions (Fig. 3-3C), the decision was made to focus on the SVZdl. This was based on it being the subregion encompassing the largest area in the p7 SVZ, and its identification as the primary site of gliogenesis in the neonatal CNS [66, 118, 119].

A small proportion of apoptotic cells in the p7 SVZdl expressed ki67 (6%, Fig. 3-5A, Fig. 3-5B). Unlike the p0 medial SVZ, the majority of dying cells in the p7 SVZdl failed to stain for any of the other cell type specific markers (72%). Of the positive

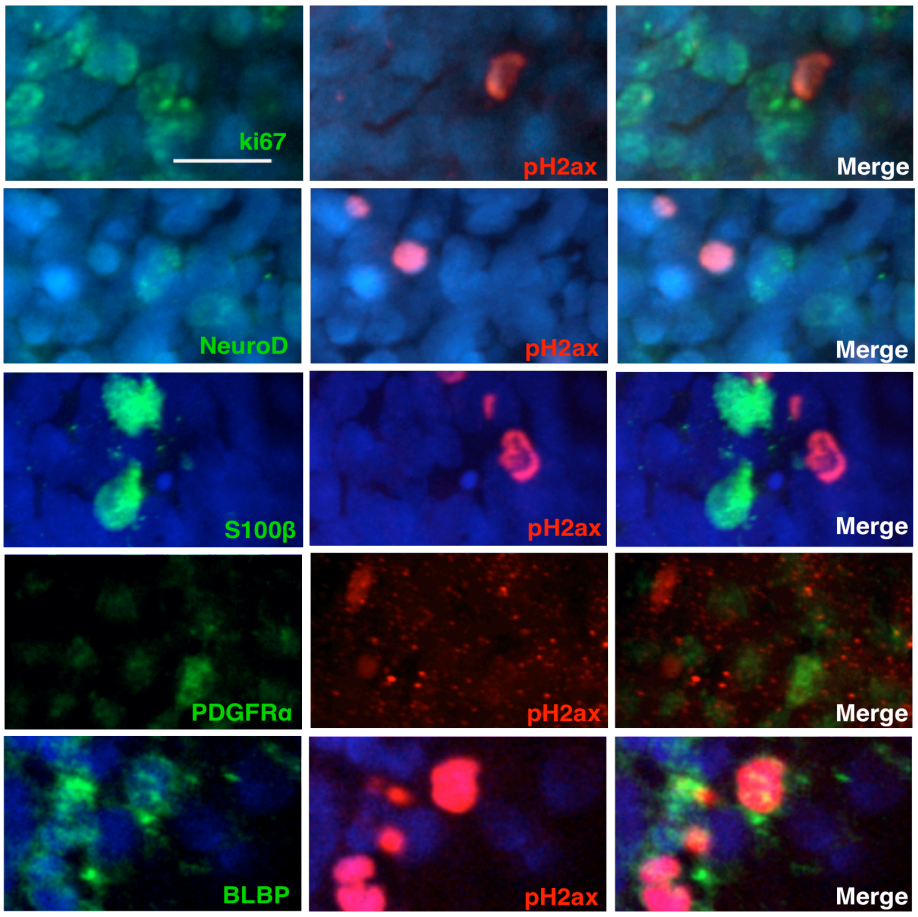
markers, BLBP was expressed by 17% of the dying cells, followed by S100B at 8%, the microglial marker, Iba-1, at 7%, PDGRα at 2%, and NeuroD at 1% (Fig. 3-5A, Fig. 3-5B).

It was interesting to observe the difference in cell types that made up the majority of dying cells in the p0 medial SVZ versus p7 SVZdl. The dying cells in the p0 medial SVZ are primarily immature neurons while the majority of dying cells in the p7 SVZdl failed to be identified. To find out if immature neurons made up a significant portion of the apoptotic population in the SVZdl at an earlier time point, the region was assessed by immunostaining with the NeuroD antibody at p0. Only 11% of the apoptotic cells expressed the immature neuronal marker (Fig. 3-5C). These results show that the SVZdl, both at p0 and p7, does not have a significant proportion of immature neurons undergoing apoptosis.

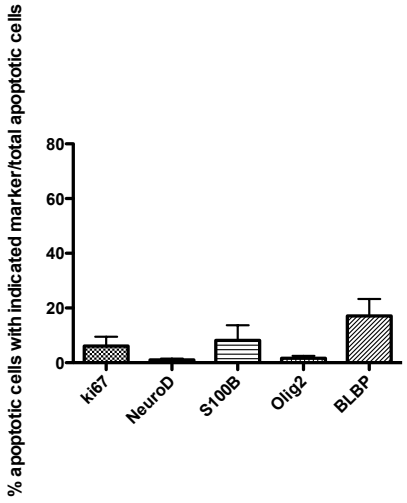
The experimental results cannot conclusively distinguish whether the differences in apoptotic cell types in the p0 medial SVZ and p7 SVZdl are due to the developmental and environmental changes over time in the neonatal SVZ and their influence in cell death of different cell populations, or are due to the SVZdl possessing unique cell population characteristics that specify different cell types to undergo apoptosis compared to other SVZ subregions. Yet, the finding that immature neurons are not eliminated in the p0 SVZdl points to the existence of different cell populations between the SVZ subregions. Future experiments identifying the apoptotic cell types in the remaining p0 and p7 SVZ subregions will help in resolving this question.

Figure 3-5.

3-5A



3-5B



3-5C

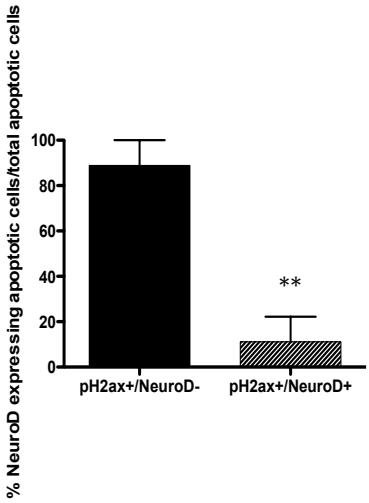


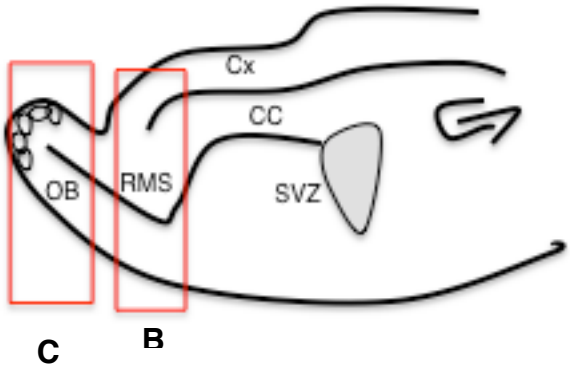
Fig 3-5: The majority of p7 SVZdl apoptotic cells do not costain for known markers **(A)** Confocal images of p7 SVZdl cell markers and pH2ax or cleaved caspase 3. Topro3 staining is shown in blue. **(B)** Fraction of p7 SVZdl apoptotic cells expressing each of the different cell type markers shown in 3-5A. The majority of apoptotic cells were unable to be identified (n=3 brains). **(C)** Proportion of NeuroD expressing pH2ax cells in the p0 SVZdl. The significant majority of pH2ax+ cells do not express NeuroD (n=3 brains). Six coronal sections per brain were assessed for each cell marker used. Error bars represent s.e.m. Scale bar, 43 μm. **$p < 0.05$.

3.2.4 Migratory Fate of Cells in the p0 Medial SVZ

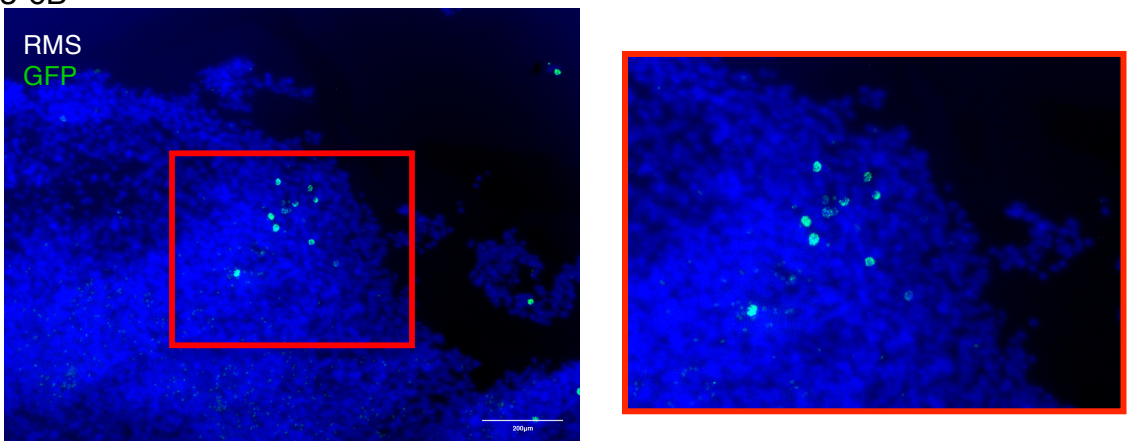
Much is known about the migratory fate of cells that reside in the p7 SVZdl. Most cells residing in this SVZ subregion differentiate into glia that migrate out and populate the cortex [123]. Less is known about the cell population located in the p0 medial SVZ, possibly due to the fact that the area significantly decreases with developmental age [124]. To determine whether cells in the medial SVZ are migratory, and if so, where their final destination is, a GFP-expressing lentivirus was stereotactically injected into the medial SVZ subregion of p0 rats (Fig. 3-6). The brains of 3, p7 animals were dissected and immunostained for GFP expression to assess the location of infected cells (Fig. 3-6B, Fig. 3-6C).

Figure 3-6.

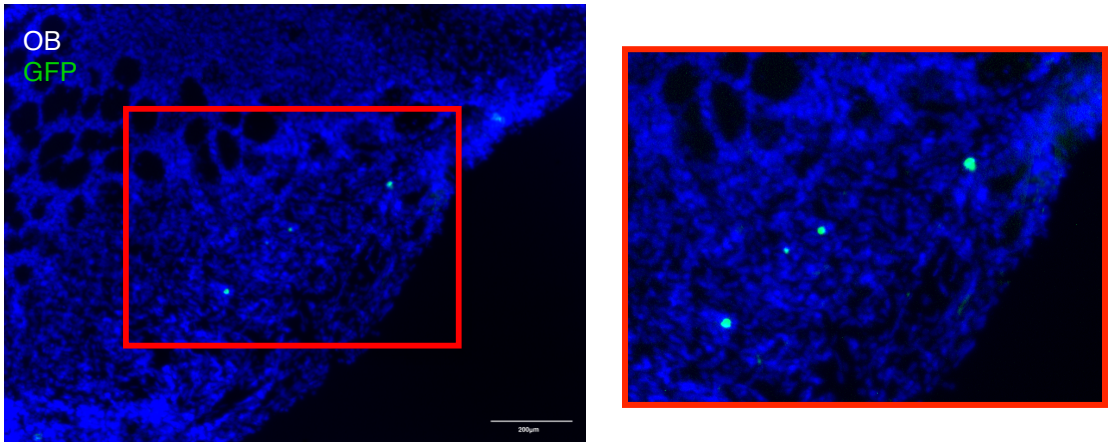
3-6A



3-6B



3-6C



3-6D

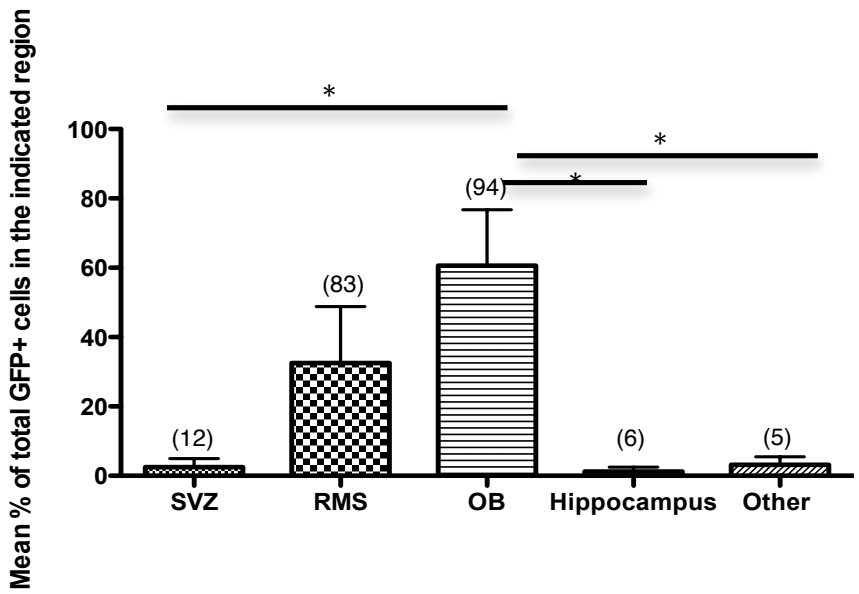


Fig 3-6. Cells in the p0 medial SVZ migrate to the RMS and OB. **(A)** Schematic drawing of sagittal section of neonatal rat brain depicting areas of GFP localization in B and C. Stereotactic injection of p0 rat pups in the medial SVZ with GFP-expressing lentivirus leads to GFP+ cells observed in the **(B)** RMS and (**C)** OB at p7. GFP is shown in green and Topro3 is shown in blue. Red inset box shows location of higher magnification panel on the right. **(D)** Proportion of GFP+ cells in each brain region is shown as a percentage of total GFP+ cells in saggital sections for each brain. Data are presented as means of these percentages ± SEM (n=3 brains). Numbers in parentheses indicate total number of GFP+ cells counted in the specific region. Scale bar, 200 μm. *$p < 0.05$.

There is a significant main effect of brain region on the distribution of GFP+ cells (Fig. 3-6D, ANOVA, $F= 6.382$, $p < 0.05$) The majority of GFP+ cells were located within the SVZ, RMS and OB, with *post hoc* multiple comparisons showing the OB having significantly higher numbers of GFP+ cells compared to the other brain regions assessed (Tukey HSD, $p < 0.05$). Based on these results, the p0 medial SVZ appears to be populated by a migratory cell population that travels via the RMS to the OB, and undergoes a significant level of cell death while still residing in the SVZ.

3.3 Discussion

This chapter has focused on describing cell death in the neonatal SVZ by investigating the level of apoptosis in various SVZ subregions, the cell types cleared and the migration patterns of cells in a specific SVZ area showing the highest level of death at p0. An important aspect of this study is that it classifies apoptosis by SVZ subregion, when most studies tend to look at the SVZ as a whole or focus on only one SVZ subregion [46, 115, 119]. This work is also noteworthy because endogenous levels of SVZ apoptosis were investigated. The majority of studies in the field have looked at neuronal cell death as a result of genetic manipulation or treatment with an apoptotic agent, which is not reflection of what could be occurring physiologically in the SVZ [70, 125, 126].

A valuable tool in collecting these results was the use of the antibody marker pH2ax. pH2ax immunostaining in the neonatal SVZ was robust, unambiguous, and easy to quantify. Employing pH2ax, along with cleaved caspase 3, in immunohistochemistry experiments lent further certainty to the classification of cell populations as apoptotic, and to the usefulness of pH2ax as a marker of developmental cell death.

The results gathered from quantifying the number of dying cells in each of the subregions, at two different time points, demonstrates the dynamic nature of the neonatal SVZ. The number of apoptotic cells is not distributed equally among the subregions at birth and the subregion with the highest amount of apoptotic cells is not consistent throughout SVZ development. At p0, the medial SVZ contains the highest concentration of dying cells, while at p7, apoptotic cells are more equally scattered

amongst the different SVZ subregions. At p7, the incidence of apoptotic cells increases in the dorsal, SVZdl, and ventral SVZ relative to their initial levels at p0.

The work of Thomaidou and colleagues had previously described the amount of cell death in the neonatal SVZ [46]. They report that on average 3% of cells in the p0 and p7 SVZ undergo apoptosis. This current work measures the density of apoptotic cells in the neonatal SVZ subregions, therefore it cannot be directly compared to the apoptotic cell proportion stated in the Thomaidou paper. There is also an existing problem with the quantification method used by the other group. The proportion of apoptotic cells reported in Thomaidou's paper is based on averaging the area of nuclear profiles in the SVZ to calculate the total SVZ cell population [46]. The issue with this method is that it does not account for inherent variation in cell areas, especially with respect to apoptotic versus non-apoptotic cells. The development and employment of a standard method for *in vivo* SVZ apoptosis quantification in the cell death research field will allow for more accurate and consistent reports of apoptosis levels in the neonatal SVZ.

The relevance of the varying amounts of apoptosis in the subregions of the neonatal SVZ during development is still unclear. One of the noticeable aspects of the SVZ in the transition from the neonate to the adult is the decrease in area of the region [127, 128]. Apoptosis could account for the decrease in subregion area, thereby making it a cellular process involved in the architectural maturation of the neonatal SVZ to the adult SVZ. Another issue related to the impact of cell death in this region, is whether apoptosis is a stochastic or cell type specific process in the neonatal SVZ subregions.

The question of cell type specific apoptosis in the SVZ was addressed via immunostaining performed at p0 and p7. Experimental results reveal two different primary cell populations being cleared. In the p0 medial SVZ, the majority of dying cells are postmitotic, as observed in the negative staining for the markers ki67 and Mcm2. The major proportion of dying cell population is further classified as immature neurons via positive staining for NeuroD and HuD. The few studies that have looked at apoptosis in the neonatal SVZ had failed to identify the majority of dying cells, but results from other studies investigating neonatal cell death in the rodent basal forebrain, dorsal lateral geniculate nucleus, and retina, confirm that the majority of dying cells fall into the neuronal lineage [129-131].

The death of immature neurons in the p0 medial SVZ could be a result of the inherent sensitivity of these cells to biological stressors. Evidence for this idea is shown in newly born neurons in various rodent brain regions undergoing a hypersensitivity period to proper levels of NMDA signaling in the first two weeks after birth [132]. Thesis chapter 5 will discuss an alternative environmental stimulus that might provoke immature neurons in the neonatal medial SVZ towards apoptosis.

Later in SVZ development at p7, significant portions of the apoptotic cells were unable to be identified by immunostaining with various cell specific markers. In contrast to the p0 medial SVZ, most p7 SVZdl apoptotic cells did not express NeuroD. It is possible that future immunostaining experiments with mature neuronal markers might identify the apoptotic cells in the p7 SVZdl. Another difference is the larger portion of p7 SVZdl dying cells expressing mitotic markers, ki67 and Mcm2. A compelling

explanation for the differences between the two time points is that the SVZ subregions host different cell populations. Even at p0, the SVZdl has few dying cells that express NeuroD.

There is the possibility that the failure for p7 apoptotic cell type verification was due to the cell's antigens being degraded during cell death. If true, then this would show a difference in the rate of apoptosis between p0 and p7 SVZ subregions, because the same antibodies used in the p7 SVZdl could detect cell marker antigens in p0 medial SVZ apoptotic cells. These findings taken together further strengthen the view that SVZ subregions are distinct structures, with each subregion having a unique cell type composition. Future experiments will determine the causes of the differences in cell type clearance in each SVZ subregion.

The limited information surrounding the neonate SVZ subregions has been mentioned in the previous thesis sections. The observation of the medial SVZ hosting the highest amount dying cells at p0 led me to select this subregion for further characterization. Determining the migration potential and destination of neonatal SVZ cells helps towards identifying the functional purpose of cells in this subregion. Studies have shown that neurons born in the neonatal SVZ have multiple potential migration destinations. The majority of SVZ cells migrate to the OB to differentiate into inhibitory neurons [71]. SVZ cells in the perinatal SVZdl also migrate to the cortex, white matter, and striatum to differentiate into glial or neuronal cells, and lateral SVZ cells migrate to the striatum to become medium-sized spiny neurons [133].

Stereotactic injection of GFP-expressing lentivirus in the p0 medial SVZ revealed

labeled cells primarily in the SVZ, RMS and OB when observed at p7. The location of the GFP+ cells indicates that a high portion of cells in the p0 SVZ medial subregion are neuronal precursors that migrate to the OB. Work from another group agrees with this current finding. Their results show the OB destination of cells from the p0 mouse medial SVZ when brains were observed one month after labeling [124]. These results highlight the migratory nature of p0 medial SVZ cells, as well as the fact that these OB destined neural precursors undergo apoptosis even before they leave the SVZ. The succeeding chapters will aim to clarify the mechanism underlying apoptosis of cells in the neonatal SVZ specifically in the medial subregion and SVZdl.

Chapter 4. Intracellular Pathways Involved in Developmental SVZ Apoptosis

4.1 Introduction

BH3-only proteins play an integral role in mediating the intrinsic apoptosis pathway. There are at least 10 identified members of the mammalian BH3-only protein family, all of which are proapoptotic and have been shown to participate in both pathological and developmental neuronal apoptosis [134, 135]. Given this information, BH3-only proteins provide a potential role in the molecular mechanism for the cell death occurring in the neonatal SVZ. This chapter will focus on two well-studied BH3-only proteins, Bim and PUMA, and their involvement in developmental SVZ apoptosis.

Amongst the BH3-only proteins, Bim and PUMA stand out as the most promising for regulating cell death in the neonatal SVZ. Both proteins share strong apoptotic activity due to their ability to bind and neutralize all antiapoptotic Bcl-2 proteins [136]. Bim and PUMA, along with the BH3-only protein Bid, have also been shown to directly promote apoptosis by binding and activating Bax and Bak [21]. These two described features of Bim and PUMA suggest a strong likelihood of cellular apoptosis occurring when both or either protein is expressed.

Although Bim and PUMA expression in the perinatal SVZ has yet to be shown, both proteins are expressed in the nervous system [137, 138]. Bim and PUMA protein are present in the immature brain with their levels decreasing into adulthood [139]. It is worth noting that the expression time course of Bim and PUMA in the cortex parallels the incidence of apoptosis in the SVZ, which also peaks in the neonate and decreases over developmental time.

The causes of apoptosis in the developing SVZ are still unknown. Studying the expression profile and activity of Bim and PUMA in the SVZ subregions can give clues to the potential triggers for cell death in this area [140]. Bim and PUMA are activated by various stimuli. Studies have shown Bim and PUMA recruitment in various neuronal cell death paradigms, including NT deprivation models. When sympathetic neurons are unable to access NGF, there is a subsequent increase in Bim mRNA and protein levels [135]. Bim knockout mice show that cell death is not completely blocked in the PNS and CNS, instead there is a time delay to when apoptosis takes place [135].

Although PUMA expression can be induced by neurotrophin withdrawal, the protein has been mostly studied in relation to its activation by p53 in DNA damage models of apoptosis [141]. In experiments that expose neural precursors to the DNA damaging agent AraC, there is a subsequent increase in PUMA mRNA and protein levels [125]. PUMA knockout mice exposed to γ-irradiation at p5 show almost no apoptosis in various brain regions, including the SVZ, when compared to their wild type littermates [142].

The experimental results detailed in this chapter are the first to characterize Bim and PUMA function in the context of the developing SVZ subregions. Additionally, the study of Bim and PUMA activity in neonatal SVZ offers an important insight into the role of these proteins during endogenous cell death. Past studies of Bim and PUMA function have mainly investigated these proteins by experimentally inducing apoptosis rather than looking at their roles during naturally occurring cell death. The experiments that will

be described focus on the role of Bim and PUMA activity *in vivo*, during the progression of SVZ development.

4.2 Results

4.2.1 Bim is Expressed in the Developing SVZ

Bim expression was assessed by immunohistochemistry and confocal microscopy in the p0 and p7 SVZ. Bim is expressed in the p0 SVZ, with more staining for the protein in the medial area compared to the other SVZ subregions (Fig. 4-1A). Counting Bim+ cells in the p0 medial SVZ reveals that about 10% of the cells express Bim. Bim protein is also present in the p7 SVZ (Fig. 4-1B). In order to confirm the specificity of the Bim antibody, frozen brain sections were treated with a Bim blocking peptide prior to immunostaining. Bim signal was completely abolished in the p0 SVZ with pre-exposure to the blocking peptide (Fig. 4-1C). Brain sections from p0 Bim knockout mice were also tested, and showed no staining with the Bim antibody (data not shown).

To determine whether there was a correlation between BIm expression and cellular apoptosis, the p0 medial SVZ and p7 SVZdl were subjected to double labeling for Bim and the apoptosis marker, pH2ax. A significant portion, 57%, of pH2ax+ apoptotic cells in the p0 medial SVZ were Bim+ compared to those that were positive for pH2ax alone (Fig. 4-1D; Student's t-test, $t=6.027$, $p < 0.005$). In contrast, only 19% of pH2ax+ apoptotic cells in the p7 SVZdl coexpressed Bim (Fig. 4-1E; Student's t-test, $t=16.09$, $p < 0.0005$).

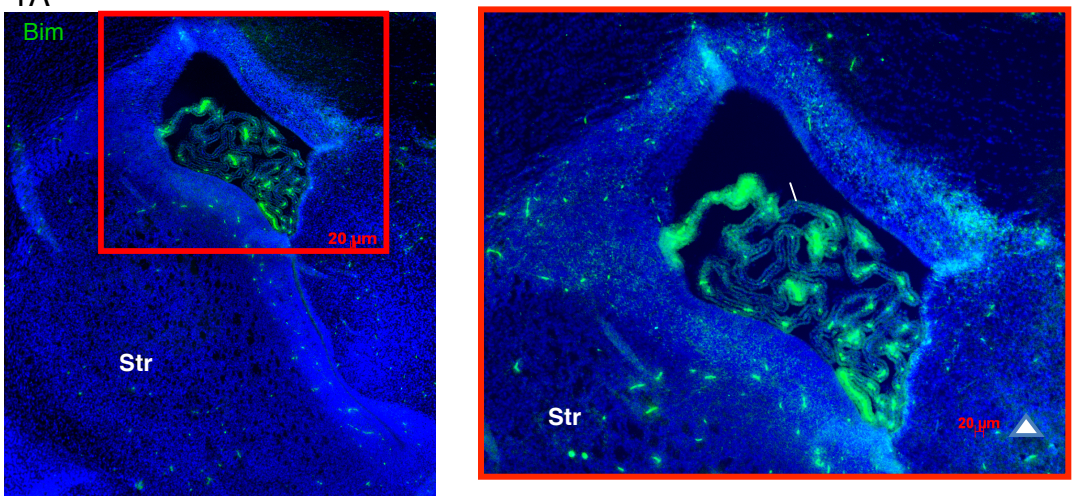
Despite the expression of Bim in the majority of dying cells in the p0 medial SVZ,

there were far more cells expressing Bim that did not have pH2ax present. An average of 94% of Bim+ cells did not express pH2ax. One reason for this observation could be attributed to the fact that Bim lies upstream of pH2ax in the apoptotic cascade [143] . pH2ax may not have been present at the time Bim was expressed in the cells. Alternatively, Bim expression alone may not be sufficient to trigger death; modification of Bim or activation of additional death proteins may be required for this event to occur.

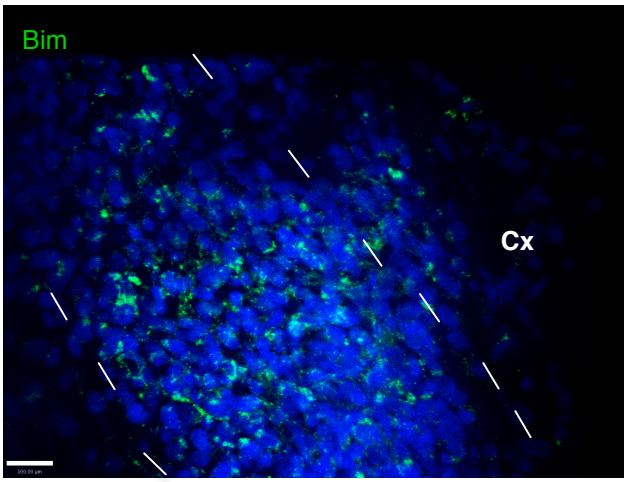
These findings indicate that Bim could be involved in apoptosis in the p0 medial SVZ. The apoptotic role for Bim may also be temporally or spatially restricted due to Bim expression primarily being found in dying cells in the p0 medial SVZ and not in the p7 SVZdl.

Figure 4-1.

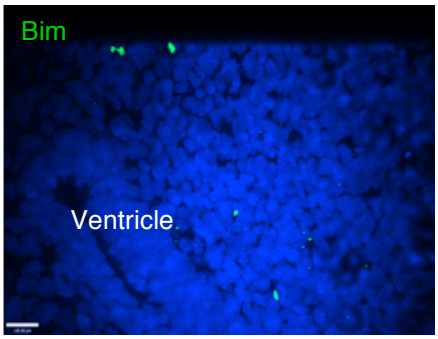
4-1A



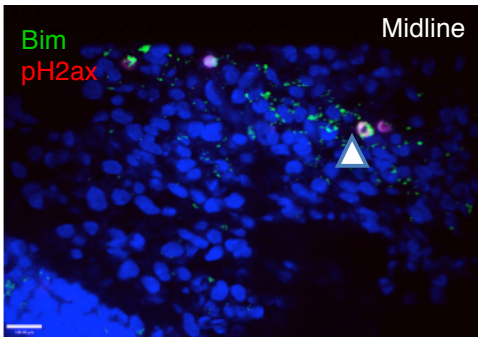
4-1B

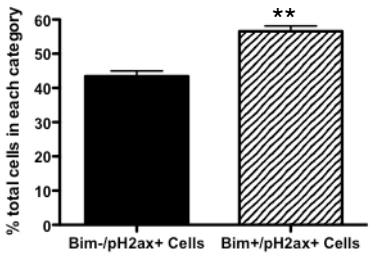

Bim
Cx

4-1C

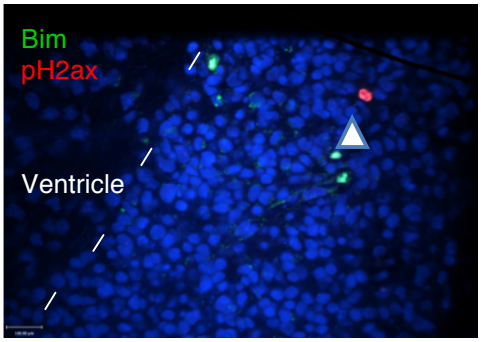

Bim
Ventricle

4-1D


Bim
pH2ax
Midline


% total cells in each category
60
50
40
30
20
10
0
**
Bim-/pH2ax+ Cells
Bim+/pH2ax+ Cells

4-1E



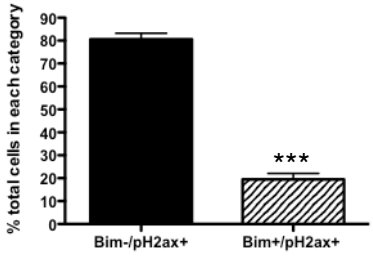


Fig 4-1. Bim is expressed in apoptotic cells in the p0 and p7 SVZ. Immunohistochemistry for Bim and pH2ax in rat frozen coronal sections. **(A)** Bim expression in the p0 SVZ. Red inset box shows location of higher magnification panel on the right. Bim expression in the medial SVZ (arrowhead). Nonspecific staining is observed in the choroid plexus. Scale bar, 20μm **(B)** Bim expression in the p7 SVZdl. White lines denote SVZdl border. Scale bar, 100μm **(C)** Specificity of Bim immunostaining was confirmed with Bim blocking antibody. Scale bar, 130 μm **(D)** Confocal image and quantification of Bim and pH2ax expression in the p0 medial SVZ. The majority of pH2ax+ cells express Bim (n=3 brains). Arrows denote representative neurons expressing both Bim and pH2ax in the p0 medial SVZ. Scale bar, 100 μm. **(E)** Confocal image and quantification of Bim and pH2ax expression in the p7 SVZdl. The majority of pH2ax+ cells do not express Bim (n=3 brains). White lines mark the SVZdl border and arrows denote representative cells expressing pH2ax alone. DAPI and Topro3 are shown in blue, Bim is shown in green, and pH2ax is shown in red in all images. All error bars represent s.e.m. Scale bar, 100 μm. **p < 0.005, ***p < 0.0005. Str, striatum; Cx, cortex.

4.2. Bim is Required for Apoptosis in Early Medial SVZ Development

The above results show the coincident expression of Bim and pH2ax in the p0 medial SVZ. Based on these findings, it was hypothesized that Bim could be required for apoptosis in this subregion at p0. Bim knockout mice obtained from the laboratory of Dr. Richard Libby (University of Rochester Medical Center) were used to test this. pH2ax immunostaining was quantified and compared in the p0 medial SVZ between

Bim knockout and wild type mice from the same genetic background and colony.

There is an 89% reduction in the density of pH2ax+ cells in the p0 medial SVZ of Bim knockout versus wild type mice (Fig. 4-2A, Student's t- test, $t=6.015$, $p < 0.0005$). Studies on the effects of Bim removal have shown that it does not offer complete protection from apoptosis; it instead delays cell death to later time points [135, 140]. In order to see if this was the case for cells in the p0 medial SVZ, pH2ax expression was measure in the p7 medial SVZ between Bim knockout and wild types. Knockouts no longer had a significant reduction in the density of pH2ax expressing cells in the p7 medial SVZ compared to wild type mice (Fig. 4-2B; Student's t-test, $t=0.9592$, $p=3918$). In fact, comparison of the density of apoptotic cells in the medial SVZ of p0 and p7 Bim null mice showed a significant increase at p7, which was not observed in wild types (Fig. 4-2C; Student's t-test, $t=2.933$, $p < 0.05$).

Immunostaining of frozen coronal brain sections was also quantified and compared between the Bim knockout and wild type p7 SVZdl. Since only a small proportion of apoptotic cells in the p7 SVZdl expressed Bim, Bim elimination was predicted to have little or no effect. Quantification of pH2ax+ cells revealed a trend for

Figure 4-2.

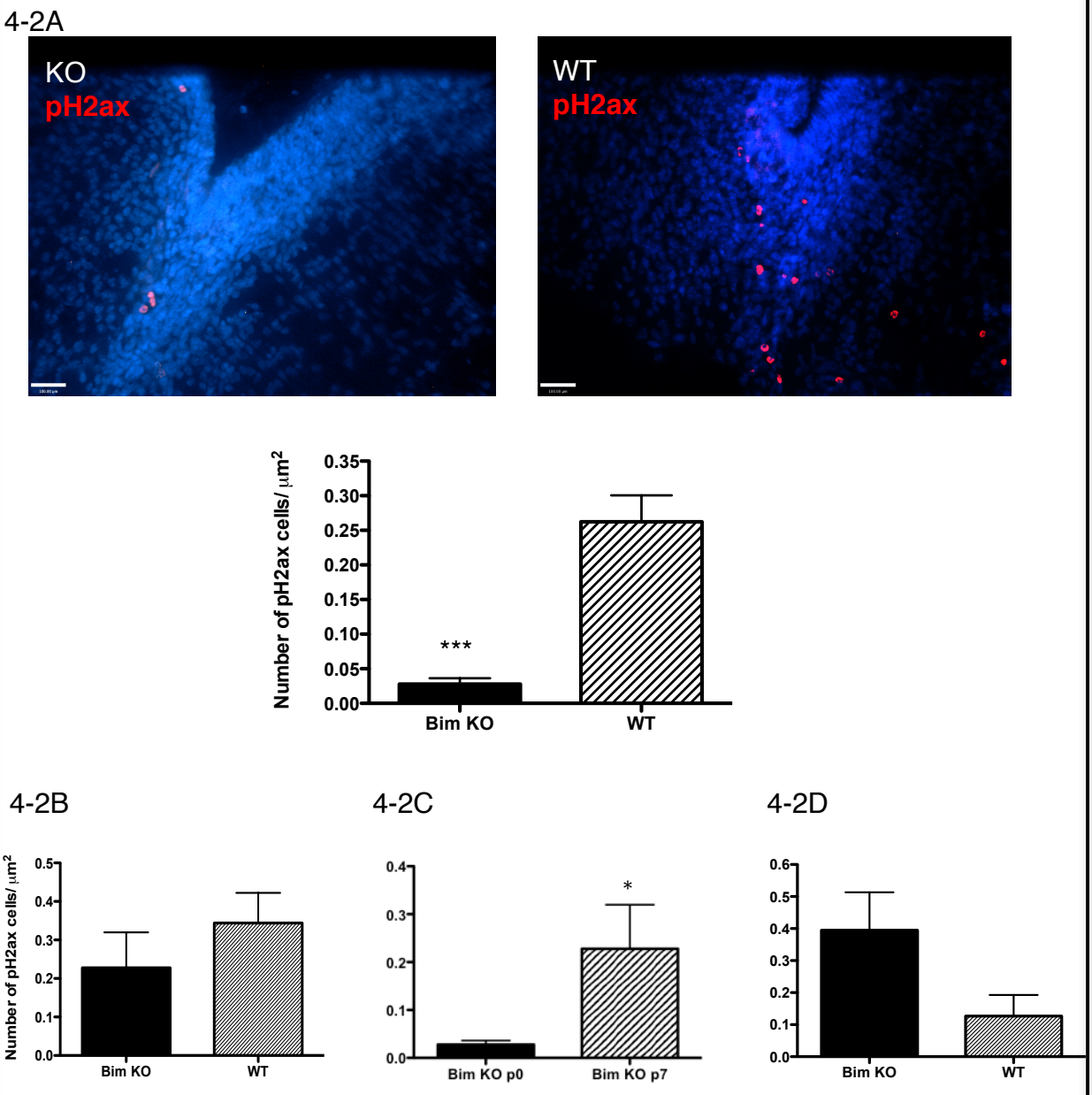


Fig 4-2. Bim is required for apoptosis in the p0 medial SVZ. **(A)** Confocal images of pH2ax antibody-labeled cells in Bim knockout (KO) and wild type (WT) p0 medial SVZ (red). Topro3 is shown in blue. Scale bar, 100 μm. All quantification was performed on mouse frozen coronal brain sections. Density of pH2ax+ cells in Bim KO and WT p0 medial SVZ (n=5 brains). **(B)** Density of pH2ax+ cells in Bim KO and WT p7 medial SVZ (n=3 brains). **(C)** Density of pH2ax+ cells in p0 and p7 Bim KO medial SVZ. There is a significant increase in pH2ax+ cells in the Bim KO p7 medial SVZ. **(D)** Density of pH2ax+ cells in Bim KO and WT p7 SVZdl (n=3 brains). All error bars represent s.e.m. ***$p < 0.0005$, *$p < 0.05$.

more apoptotic cells occurring in the p7 SVZdl of Bim knockout versus wild type mice, but this difference did not reach significance (Fig. 4-2D; Student's t-test, t=2.716, p=0.1557).

These results taken together show the specific presence and requirement of Bim in early SVZ apoptosis in the p0 medial SVZ.

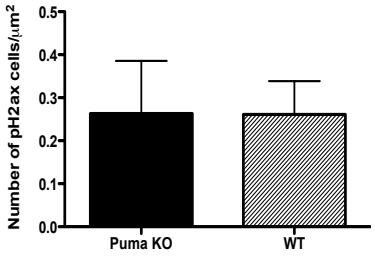
4.2.3 Loss of PUMA Does Not Appear to Affect Apoptosis during p0 Medial and p7 SVZdl Development

Studies have shown the requirement of PUMA for irradiation-induced apoptosis in the neonatal SVZ [142]. The effect of PUMA elimination on the unperturbed SVZ has not been addressed. PUMA knockout mice from the same genetic background and colony were also obtained from the laboratory of Dr. Richard Libby, and were used to investigate whether the presence of PUMA was important for p0 medial SVZ and p7 SVZdl apoptosis. Immunostaining and quantification of pH2ax in the p0 medial SVZ of PUMA knockout and wild type mice revealed no significant difference in cell death numbers between the genotypes (Fig. 4-3A; Student's t-test, t=0.1525, p=0.9881). Examination of pH2ax in the p7 SVZdl of PUMA knockout and wild type mice also showed no significant difference in the number of apoptotic cells, despite there being a trend towards less pH2ax+ cells in the PUMA knockout (Fig. 4-3B; Student's t-test, t=1.773, p=0.0966).

The lack of any significant effect of PUMA deletion on SVZ apoptosis in the p0 medial and p7 SVZdl indicates that PUMA is not essential for cell death at these observed time points in these SVZ subregions.

Figure 4-3.

4-3A



4-3B

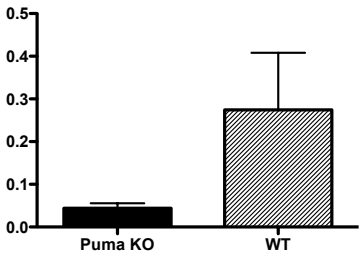


Fig. 4-3. PUMA is not required for apoptosis in the p0 medial SVZ and p7 SVZdl. Quantification of pH2ax expressing cells in mouse frozen coronal brain sections **(A)** p0 PUMA KO and WT medial SVZ pH2ax density (n=6 brains). **(B)** p7 PUMA KO and WT SVZdl pH2ax density (n=3 brains). All error bars represent s.e.m.

4.3 Discussion

The results presented provide a better understanding of the relevance of two BH3-only proteins, Bim and PUMA, in cell death in the developing SVZ, specifically in the p0 medial SVZ and the p7 SVZdl. This is the first study to focus on the roles of Bim and PUMA in the context of the neonatal SVZ, a neurogenic and gliogenic zone in the postnatal brain, and also offers a description of Bim and PUMA actions in non-induced neuronal cell death.

One of the first findings from these experiments was evidence for the expression of Bim in the neonatal SVZ. Bim expression was shown via immunohistochemistry in the p0 and p7 SVZ. High expression of Bim was particularly notable in the p0 medial subregion, where apoptosis is at its peak level at this time period. Loss of Bim signal in the p0 medial SVZ with application of a Bim blocking antibody, and the absence of

staining in the Bim null mouse indicate the specificity of the Bim antibody for its target. It is important to note that the Bim antibody used detects all three Bim isoforms, BimEL, BimL, and BimS. It is unknown whether a specific Bim isoform is preferentially expressed in the p0 and p7 SVZ or if all three isoforms are equally present. Results showing the predominant expression of BimEL in the CNS and its specific involvement in retinal developmental apoptosis make it the likely Bim isoform expressed in the neonatal SVZ [137, 140]. Future studies can determine this using antibodies specific for BimEL to probe for its expression in the p0 and p7 SVZ.

After the presence of Bim in the neonatal SVZ was established, the link between Bim and apoptosis in this region was investigated. The correlation of Bim expression with apoptosis was shown by the presence of Bim in the majority of pH2ax+ cells in the p0 medial SVZ (Fig. 4-1D). Bim induction during neuronal apoptosis had previously been noted in a number of *in vitro* studies [135, 144-146]. Cell culture studies of superior cervical ganglion and cerebellar granule neurons have demonstrated Bim induction in apoptotic cells after NGF deprivation [135]. The coincidence of Bim and pH2ax in the p0 medial SVZ could indicate a similar induction during normal apoptosis *in vivo*.

Although the majority of pH2ax+ cells in the p0 medial SVZ coexpressed Bim, there were far more Bim+ cells that did not express the apoptotic marker. It was mentioned above that chronologically, Bim expression occurs before pH2ax, and this could explain the nonoverlap of the markers. There are still other potential explanations worth discussing. The issue of Bim sufficiency to induce cell death in the neonatal SVZ

is one of them.

In vitro experiments have shown that cells transfected with Bim rapidly undergo apoptosis [145, 147]. Yet the ability of Bim alone to induce cell death may be different *in vivo* in the p0 medial SVZ. The sufficiency of Bim to induce neonatal SVZ apoptosis could be dictated by a Bim protein threshold that must be reached. Studies have shown low Bim expression in healthy neurons, and that Bim levels become elevated when a strong apoptotic stimulus is presented [135, 137]. It is also possible that Bim may need to interact with another BH3-only protein, such as tBid, before it can cause apoptosis [21]. Finally, Bim protein present in pH2ax- cells could have undergone post-translational modification, which would affect its proapoptotic activity. Bim phosphorylation has two different outcomes depending on the signaling pathway that induced the phosphorylation. Phosphorylation via the ERK signaling pathway renders Bim unstable and susceptible to degradation [148]. In contrast, phosphorylation via the JNK pathway potentiates the proapoptotic activity of Bim [148]. Bim+/pH2ax- cells in the p0 medial SVZ may either have Bim phosphorylated by the ERK pathway, thereby sentencing Bim to undergo eventual degradation, or have Bim lack phosphorylation from the JNK pathway, and remain inefficient at promoting apoptosis. Follow up experiments should assess p0 medial SVZ cells for Bim post translational modification, and differentiate between these two phosphorylation possibilities, as well as look at the expression of other BH3-only proteins in the neonatal SVZ and measure their contributions to apoptosis.

Examination of Bim-pH2ax expression in the p7 SVZdl revealed Bim to have a

contrasting expression pattern when compared to the p0 medial SVZ. The coexpression of Bim and pH2ax was not observed, with Bim being undetected in the majority of pH2ax+ cells. The differences observed in the coexpression of Bim and pH2ax in the p0 medial SVZ and p7 SVZdl could be a product of the changes in Bim as a requirement of apoptotic cells that occur in SVZ cell populations over time or differences in the influence of Bim on apoptosis between cell populations within the different SVZ subregions.

Since Bim was expressed in the majority of dying cells in the p0 medial SVZ, its requirement in the apoptotic process was tested with the use of Bim knockout mice. The majority of mice lacking Bim die embryonically, but the surviving offspring are healthy [149]. Bim knockout mice showed significantly decreased apoptosis in the p0 medial SVZ compared to wild type mice. This result points to the requirement of Bim for apoptosis in this SVZ subregion at p0. The protective effect of Bim elimination has been observed in other neuronal populations *in vivo* and *in vitro* [135, 140]. Bim knockout mice display temporary decreased apoptotic death in sympathetic, cerebellar and retinal neuronal populations compared to wild type littermates [135, 140].

There was no significant difference in the number of apoptotic cells between Bim knockout and wild type mice in the p7 SVZdl, only a trend towards higher apoptosis in the SVZdl of Bim knockout mice. Another observation was the significant increase in the number of pH2ax+ cells in the p7 versus p0 medial SVZ in Bim knockout, but not wild type mice. These are reminiscent of reports made by other research groups of the transient protective quality of Bim elimination. Studies of the retina of Bim knockout mice

reveal increased numbers of apoptotic retinal ganglion cells at later time points compared to wild types [140]. The idea of a transient protective role of Bim elimination in the SVZ should be further explored as an explanation for the increase of SVZ apoptosis in Bim knockout mice at later time points compared to wild types. For example, apoptotic cells should be quantified in the p0 SVZdl of Bim knockout mice to confirm whether the SVZdl experiences the same increase in dying cells as the medial SVZ between p0 and p7 when Bim is removed.

The role of PUMA in p0 medial SVZ and p7 SVZdl apoptosis was also investigated in this chapter. PUMA was chosen for these studies, because along with Bim, it possesses strong apoptotic activity. PUMA knockout mice have shown decreased TUNEL staining in the adult hippocampal dentate gyrus compared to their littermate controls [150]. PUMA mRNA is also downregulated in the SVZ when ischemic rat brains are treated with a p53 inhibitor that works to reduce apoptosis, indicating that PUMA could be involved in initiating apoptosis in ischemic-induced cell death in the brain [151]. The ability of PUMA to promote cell death in the hippocampus, a neurogenic area in the postnatal brain, and its presence in the adult SVZ during trauma, begs the question if it is also present in the neonatal or adult SVZ under physiological conditions and if it has a function in apoptosis in this region.

PUMA expression was unable to be directly determined due to the lack of a specific PUMA antibody for immunohistochemistry. The unknown presence of PUMA in the SVZ makes it difficult to explain the lack of a significant difference in the numbers of pH2ax expressing cells between PUMA knockout and wild type mice in the p0 medial

SVZ and p7 SVZdl. The p7 SVZdl does, however, display a trend towards less apoptotic cells in the PUMA knockout versus wild type mice. The reasons for the similarity in apoptotic cells numbers in PUMA knockout and wild type mice could be, but are not limited to, PUMA not being expressed in the p0 or p7 SVZ or PUMA being present but unnecessary for apoptosis to occur in the p0 medial and p7 SVZdl due to compensation by another BH3-only protein.

In summary, Bim and not PUMA has been shown to have a role in developmental cell death in the SVZ. Bim is necessary for maximal apoptosis in the p0 medial SVZ. However, the effects of Bim elimination appear to be limited, with Bim only delaying cell death. Future experiments should investigate the temporary protective effect of Bim removal during SVZ developmental apoptosis as well as PUMA expression in the neonatal SVZ. They should also evaluate whether loss of Bim results in compensation by other BH3 proteins.

Chapter 5. Extracellular Signaling Involved in Developmental SVZ Apoptosis

5.1 Introduction

Numerous studies have implicated NTs in many aspects of SVZ cellular activity, such as proliferation, cell survival and migration [152]. A thorough investigation into the extrinsic mechanisms influencing apoptosis in the developing SVZ includes looking at the effects of NT signaling in the region. Much of the work studying the significance of NT signaling in the SVZ has been done in the adult and the embryo, with only a handful of studies concerning the neonatal SVZ. Currently there have been reported roles for NGF, BDNF and NT-3, along with their respective receptor partners TrkA, TrkB, TrkC and p75 in SVZ development [107, 153, 154]. Out of this group, p75, TrkB and BDNF have been the most heavily studied, and have emerged as the most likely candidates in regulating perinatal SVZ cell death.

The p75 receptor interacts with all NTs with equal binding affinity, and is expressed in the neonatal SVZ, specifically in the p2 SVZdl [92, 153]. p75 has been primarily studied with regard to its influence on SVZ neurogenesis, and its effects on neonate apoptosis are largely unknown. This is despite the fact that p75 activation is known to promote apoptosis in certain cellular contexts, specifically when Trk receptors are not coexpressed with p75 in cells [89]. The presence of p75 in the developing SVZ and its known association with apoptosis make it worth further investigation as a potential regulator of apoptosis in the neonatal SVZ.

As for TrkB and BDNF, their widespread expression in nervous tissue speak to the inherent role the two play in various neuronal processes. TrkB is the most abundant

receptor and BDNF the most widely expressed NT in the nervous system [155]. *In vitro* and *in vivo* studies looking at the effects of TrkB signaling show it promoting survival as well as proliferation in the SVZ. Adult rat SVZ derived cell cultures treated with BDNF, and adult rats infused with BDNF in the LV show an increase in cell survival [126, 156].

Complementing the BDNF studies are results from TrkB and BDNF knockout mice. Ernfors and colleagues bred and assessed BDNF null mice for apoptosis using TUNEL staining and reported no difference in the amount of apoptosis in the SVZ of knockout versus wild type mice until p13 [157]. In contrast, TrkB null mice showed increased TUNEL staining in various brain regions, including the SVZ and OB, compared to wild type mice as early as p0 [158, 159].

Elimination of TrkB or BDNF affects SVZ apoptosis at different time points, with TrkB nulls showing increased apoptosis earlier than BDNF knockout mice [159]. This difference could relate to the fact that TrkB may still be activated in the absence of BDNF via NT-4, leading to a longer time of survival signaling compared to the TrkB null mice. The blockade of TrkB receptor alone would be a better test of the necessity of TrkB signaling in the neonatal SVZ.

Baydyuk and colleagues used mice that had TrkB selectively deleted in striatal neurons, in order to further investigate the implications of TrkB signaling in striatal neuron development [115]. Along with their later study employing BDNF knockout mice, they found a significant increase in apoptotic incidents in a premigratory population of cells in the p0 lateral SVZ [40]. The limitations of these studies are that apoptosis

changes were only measured in the lateral SVZ and not extended to the remaining SVZ subregions, and that TrkB and BDNF elimination were not specific to the SVZ alone.

The role of TrkB cannot be generalized to the entire SVZ, because only the lateral SVZ was investigated. Chapter 3 highlighted the differences in apoptotic cell density amongst the SVZ subregions at p0 and p7. These differences are evidence that there is too much variation between SVZ subregions for results from one subregion to be extended to the rest. The other limitation with the experiments performed with the TrkB and BDNF nulls is that TrkB and BDNF was not selectively eliminated in the SVZ cell population. There was an observed decrease in TrkB protein in the cortex and the striatum of the TrkB knockout mice [115]. It cannot be ruled out that loss of TrkB receptors and BDNF in other brain regions could have indirect consequences on SVZ apoptosis.

There is still a need for more studies looking at the relevance of TrkB signaling in the developing SVZ. It is difficult to make strong conclusions on the influence of TrkB signaling on cell survival in the SVZ from the TrkB and BDNF knockout data alone. Conducting experiments focusing on other SVZ subregions would allow one to come to a better understanding on the relationship between TrkB signaling and vulnerable cell populations in the neonatal SVZ. In addition, the stereotactic injections that will be described in this chapter alter TrkB signaling locally in the SVZ, without affecting TrkB receptors in other regions of the brain. The localization of TrkB disruption avoids the potential confounding influence of TrkB disruption in other brain structures affecting apoptosis in the SVZ.

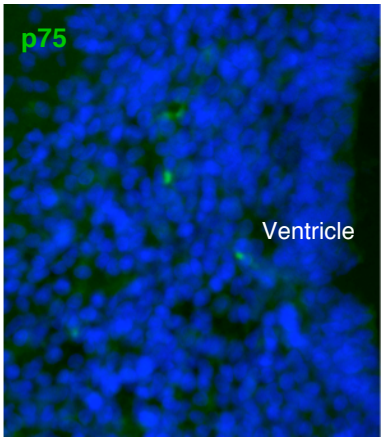
The results presented in this chapter note the presence and activity of TrkB in the p0 medial SVZ and p7 SVZdl and the effects of acute interference of TrkB signaling on p0 medial SVZ apoptosis levels. This chapter gives a detailed account of TrkB signaling in the developing SVZ, revealing an immature, premigratory population of SVZ cells destined for the OB that is influenced by NT signaling.

5.2 Results

5.2.1 TrkB Receptor Signaling is Necessary for Cell Survival in the Developing SVZ

The p0 medial SVZ showed a significant amount of death compared to all other SVZ subregions (Fig. 3-2C). To find out whether p75 could be regulating cell death in this region at p0, p75 expression was assayed via immunohistochemistry on frozen rat coronal brain sections with a p75 specific antibody. p75 was not detected in the p0 SVZ, but expression was observed at a later time point, p4 (Fig. 5-1A, Fig. 5-1B). Due to its apparent absence in the p0 SVZ, p75 signaling can be inferred to not be directly involved in the high density of apoptotic cells in this region.

Figure 5-1.
5-1A



5-1B

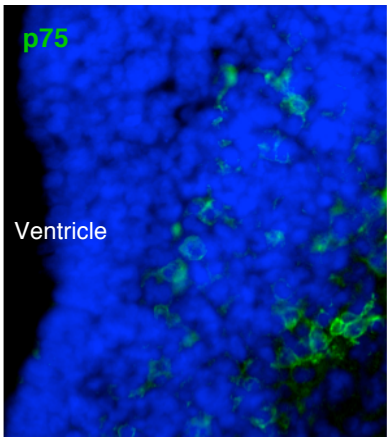


Figure 5-1. p75 is not detectably expressed in the p0 medial SVZ. Immunohistochemistry on p0 and p4 rat frozen coronal brain sections. p75 is shown in green and Topro3 is shown in blue. **(A)** Confocal image of p75 immunostaining in the p0 medial SVZ. p75 expression was not detected. **(B)** Confocal image of p75 immunostaining in the p4 medial SVZ. p75 expression was observed in multiple medial SVZ cells. Scale bar, 100μm.

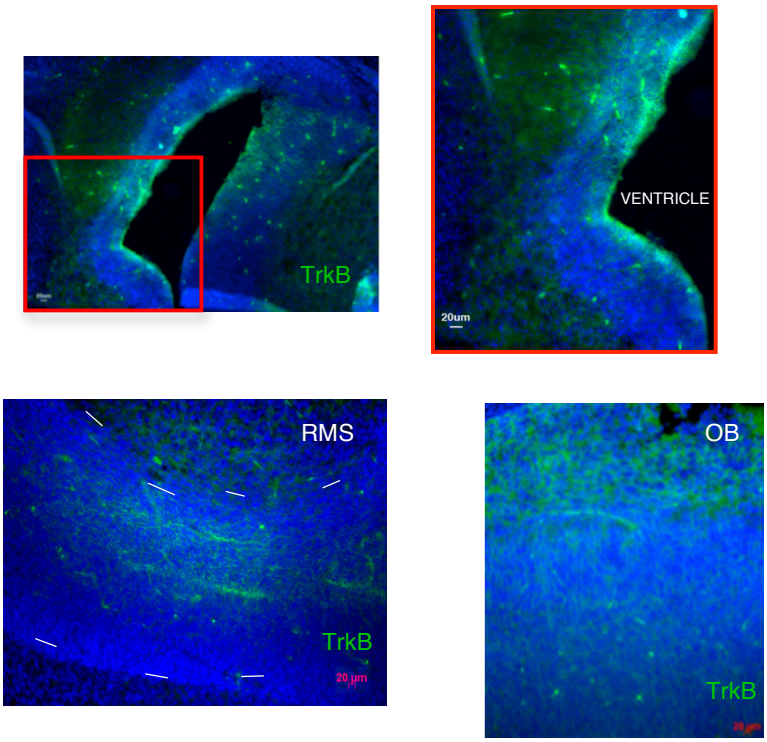
TrkB expression has been reported in the adult SVZ and inferred in the neonate from results of increased apoptosis in p0 TrkB null mice [158, 160]. In order to directly assess the role of TrkB signaling in the survival of cells in the p0 medial SVZ, TrkB expression was investigated through immunohistochemistry on rat frozen coronal brain sections with a TrkB specific antibody, which recognizes the full and truncated TrkB isoforms. Results show TrkB expression in all subregions of the p0 SVZ (Fig. 5-2A). TrkB was also present in the p0 RMS and OB, indicating TrkB signaling could promote the survival of migrating neuroblasts and other cells that lie in the migratory path (Fig. 5-2A).

Chapter 3 measured and characterized SVZ subregion apoptosis at p7. Given the presence of TrkB at p0, it was of interest to see if TrkB was also present at p7. The same TrkB specific antibody used in p0 SVZ immunostaining was employed, and it also revealed TrkB expression in the p7 SVZ (Fig. 5-2B). The expression of TrkB at p7 implies that TrkB may have a role in SVZ development over a wide range of neonatal ages.

Once bound by its ligand, the TrkB receptor undergoes autophosphorylaton at specific tyrosine residue sites in the intracellular domain [161]. TrkB phosphorylation precedes and is required for the downstream signaling pathways that mediate the various cellular processes associated with the receptor [101]. In order to test the presence of endogenously activated TrkB receptors in the SVZ, p0 brain sections were immunostained with a phosphoTrkB (pTrkB) antibody specific to the phosphorylation of TrkB at tyrosine 816. There was extensive pTrkB immunoreactivity detected in the p0 SVZ (Fig. 5-3). However, the medial SVZ and SVZdl did not have uniform pTrkB expression, with some areas in the SVZdl and medial SVZ lacking any detectable pTrkB signal (Fig. 5-3). This indicates that there is active TrkB signaling in the p0 SVZ, although not uniform. Similar pTrkB staining has also been found in the adult SVZ, showing that TrkB signaling continues into adulthood [160].

Figure 5-2.

5-2A



5-2B

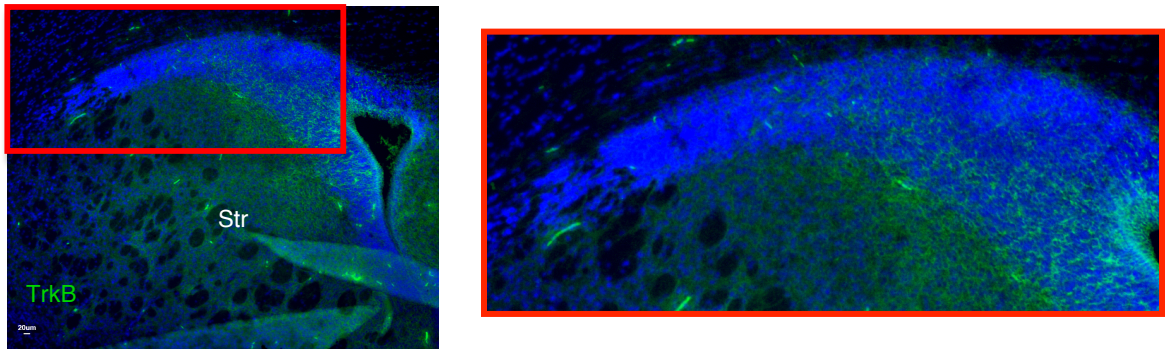


Fig 5-2. TrkB is expressed in the p0 and p7 SVZ. Immunohistochemistry on p0 and p7 rat frozen coronal sections. TrkB is shown in green and DAPI is shown in blue. **(A)** Images of TrkB expression in the p0 SVZ, RMS and OB. Red inset box shows higher magnification panel on the right. White lines mark RMS border. **(B)** Images of TrkB expression in the p7 SVZ. Red inset box shows location of higher magnification panel on the right. Scale bar, 20 μm. Str, striatum.

Figure 5-3.

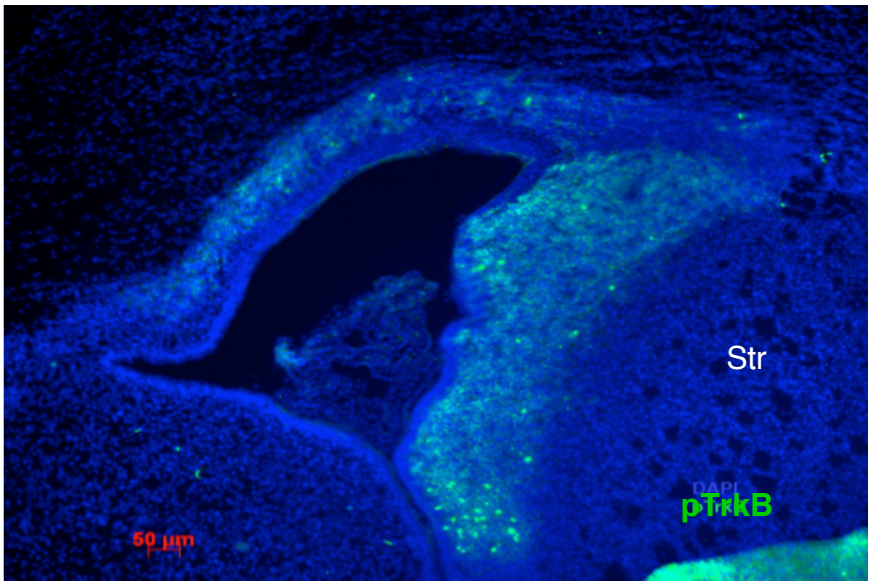


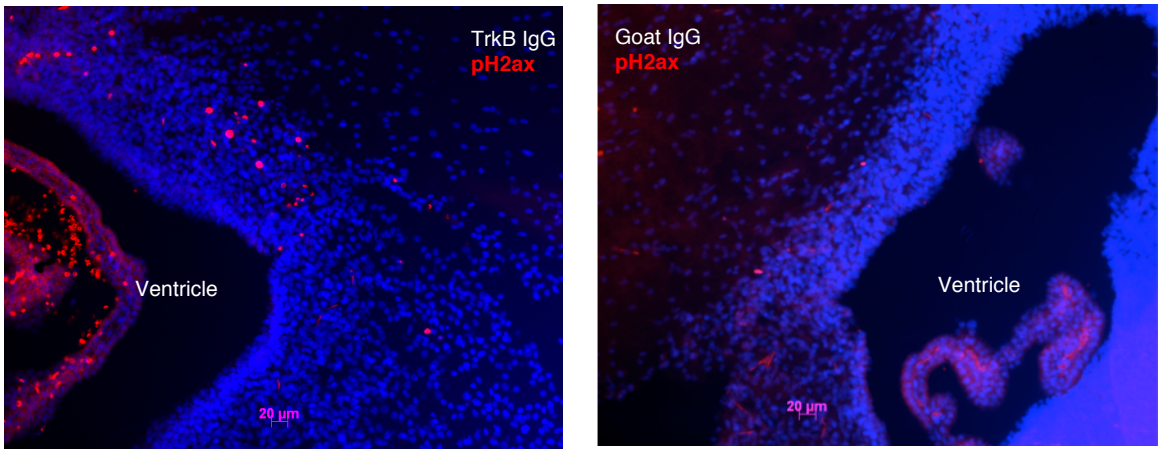
Fig 5-3. The p0 SVZ possesses endogenous TrkB receptor activity. p0 rat frozen coronal brain sections were immunostained for pTrkB. Image of pTrkB receptor antibody labeled cells in the p0 SVZ (green). DAPI is shown in blue. Scale bar, 50 μm. Str, striatum.

Based on the expression pattern of TrkB and its activated form in the SVZ, as well as its known role in supporting survival in the CNS, TrkB signaling was hypothesized to be necessary for the survival of cells in the neonatal SVZ. To test this hypothesis 50 μg/ml of either a TrkB blocking antibody or control Goat IgG antibody was stereotactically injected into the p0 medial SVZ. The injection concentration used blocks over 90% of TrkB binding, and injections tested at lesser concentrations did not yield any effect on apoptosis in the p0 medial SVZ. One day later, neonatal rat pups injected with the TrkB blocking antibody showed a significant increase, 37%, in apoptosis density in the medial SVZ compared to controls (Fig. 5-3; Student's t-test, $t=9.072$, $p <$

0.005). These results indicate that TrkB possesses a necessary role in mediating cell survival of some cells in the neonatal SVZ.

Figure 5-4.

5-4A



5-4B

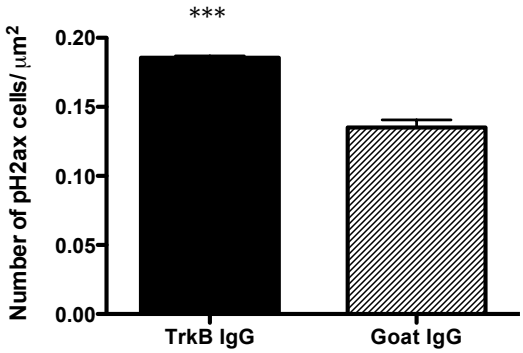


Fig 5-4. TrkB mediates p0 medial SVZ cell survival. **(A)** Immunostaining for pH2ax on TrkB IgG and Goat IgG control injected brains (red). DAPI is shown in blue. **(B)** Quantification of pH2ax expressing cells in the p1 medial SVZ of rats injected with TrkB IgG blocking antibody or Goat IgG (n=3 brains). TrkB IgG injected brains show a significant increase in apoptotic cells. Error bars represent s.e.m. Scale bar, 20µm. ***$p < 0.005$

5.3 Discussion

Several intracellular components underlying apoptosis in the neonatal SVZ have been examined in the previous thesis chapter. In particular, the roles of the BH3-only proteins Bim and PUMA have been described in context of cell death in the p0 medial SVZ and p7 SVZdl. The experiments in this chapter supplement knowledge of the intracellular pathways involved in neonatal SVZ apoptosis by elaborating on the potential extracellular signaling mechanism that selects cells to die in the p0 medial SVZ. TrkB has been revealed to be expressed, activated and necessary for mediating survival in the neonatal medial p0 SVZ.

TrkB was a promising candidate for research based on numerous studies linking the receptor activation to adult SVZ cell survival in the rodent [126, 157, 158, 160]. TrkB and pTrkB expression was shown here in the p0 and p7 SVZ as well as the p0 RMS and OB. Detection of pTrkB, the activated form of TrkB, in the SVZ indicates the presence of the full functional form of the TrkB receptor. The same data cannot, however, rule out the coexistence of the truncated form of the TrkB receptor in the neonatal SVZ. Another limitation of the study was in the identification of the cells expressing TrkB. TrkB+ cell types in the SVZ were not identified due to the difficulty of doing reliable colocalization studies with the TrkB and pTrkB antibodies employed for these studies. There have been conflicting reports as to the identity of TrkB+ cells in the adult SVZ, with some studies showing TrkB exclusively expressed by neuroblasts and others showing TrkB expression being limited to astrocytes [158, 160]. Future experiments using better experimental tools would determine the specific cell types that

express TrkB in the neonatal SVZ.

The presence of p75 was also tested in the p0 medial SVZ to see if it could, along with TrkB, have a role in survival or apoptosis in the region. p75 was not found in the p0 medial SVZ, but expression did occur at the other time point inspected, p4. Other groups have shown the expression of p75 in the rat neonate SVZ as early as p2 in SVZdl stem cells [153]. It would seem that p75 expression in the SVZ occurs very soon after p0. The differential expression of p75 is another example of the unique variability of the developing SVZ subregions. An investigation of the effects of acute inhibition of p75 in the SVZ subregions at p2 and later would lend information to the pro or antiapoptotic effect of this receptor in the developing SVZ proliferative environment.

Finally, results from functional experiments using stereotactic injection of a TrkB blocking antibody in the p0 medial SVZ showed increased apoptosis in the region compared to rats injected with a control Goat IgG antibody. These findings support TrkB signaling in the p0 medial SVZ acts to promote cell survival, and could indicate that NT signaling supports the survival of immature, premigratory neuronal populations. Experiments in the future should investigate the expression pattern of pTrkB and pH2ax in the p0 medial SVZ to test whether cells where TrkB signaling is occurring are spared from apoptosis by being pH2ax-. It will also be interesting to see if the effects of TrkB signaling are maintained in other SVZ subregions at p0 and p7. Similar results to the p0 medial SVZ would speak to the conserved survival role of the receptor throughout SVZ development.

Chapter 6. Conclusions and Future Directions

6.1 Apoptosis and the Developing SVZ

6.1.1 Apoptosis is a Hallmark of the Developing SVZ

Some of the most important insights of this thesis project are rooted in its focus on the neonatal SVZ, and the process of developmental apoptosis that occurs in this region. The SVZ is an active area of research in the field of neuroscience, yet cell death in the neonate has been insufficiently uncharacterized. Almost all of the studies investigating cell survival in the SVZ have focused on the adult [119, 158, 160, 162]. A clear understanding of the events in SVZ development is essential, because the processes occurring in the perinatal SVZ will ultimately affect normal brain function.

The experimental results from this thesis work show apoptosis occurring in the SVZ in the first postnatal week, and report for the first time an early preference in cell death for specific SVZ subregions that disappears with SVZ maturity. The reason behind the significantly higher number of apoptotic events in the p0 medial SVZ as well as the change in the pattern of apoptosis at p7 amongst the SVZ subregions is still open for speculation. One compelling explanation is the p0 medial SVZ possessing a unique population of cells, which is not found in other SVZ subregions at p0 or later on, that must be eliminated. The immature neurons undergoing apoptosis in the p0 medial SVZ may be targeted for apoptosis based on the type of mature neuron they will differentiate into in the OB.

Related to this hypothesis is the suggestion of Levison and colleagues that apoptosis in the juvenile SVZ serves to eliminate specific cell types that have been

produced during an inappropriate developmental time window and to maintain a specific cell type ratio [118]. Chapter 3 gave a detailed account of the migration of cells in the p0 medial SVZ to the OB. The p0 mouse medial SVZ has been further characterized and shown to produce the majority of the periglomerular neurons and very few of the granule neurons in the OB [124]. This finding and that from other studies have revealed that SVZ neuroblasts are not identical and possess different intrinsic properties [163, 164]. It is possible that apoptosis in the p0 medial SVZ serves as a selection mechanism, eliminating cells that are not destined to become periglomerular cells in the OB. Future experiments will investigate how the increase or decrease in p0 medial SVZ apoptosis affects the cell type ratio in the OB.

One final important consideration to keep in mind with the levels of neonatal SVZ cell death reported here is that the density of apoptotic cells may be higher in reality. The potential underestimation of apoptosis numbers in the p0 and p7 SVZ relates to the observation that apoptotic cells are cleared 1-2 hours from the time apoptosis commences [47]. There is a narrow time window for cell death antigens to be available for antibody detection during apoptosis. Therefore, the proportion of apoptotic cells reported in this thesis do not directly translate into the actual total cell loss, rather it offers a limited snapshot of ongoing endogenous cell death in the neonatal SVZ.

6.1.2 Apoptosis Targets a Premigratory Neuronal Population

p0 Medial SVZ. Immunohistological results from the experiments reported here reveal that the primary cell type cleared in the p0 medial SVZ are postmitotic immature neurons. The postmitotic neurons residing in the medial SVZ cells are premigratory, with

the majority of cells traveling to the OB via the RMS. These findings are noteworthy for several reasons, one being that they describe the death of a postmitotic population in the neonatal SVZ. SVZ apoptosis has been classified as proliferative cell death, whereby most dying cells are mitotic precursors, with postmitotic cell death occurring only after a cell had reached its final destination, [42]. Results from adult Bax/Bak knockout mice, in which the most salient phenotype was an overaccumulation of cells in the SVZ, confirmed that the primary overproduced cell type were GFAP+ stem cells and neural precursors [70].

The data from this thesis contradicts the generalization of apoptosis in the neural proliferative zones as only targeting cycling progenitors. Results here have shown that in the case of the p0 medial SVZ, the majority of dying cells are postmitotic immature neurons. These findings are also the first to highlight the apoptotic cell type in the neonatal medial SVZ subregion. The incongruous results between this report and the work of Lindsten and colleagues on SVZ cell types undergoing apoptosis could be rooted in the difference in the time points investigated, the neonatal versus adult SVZ. Only apoptotic cell types of the p0 medial SVZ and p7 SVZdl were investigated in this thesis work. Future experiments should characterize the dying cell types in the other SVZ subregions at different neonatal time periods.

Another important insight from results collected in the p0 medial SVZ is that they characterize a cell population that undergoes apoptosis before they reach their final destination, and before they synapse with a target. The neonatal SVZ adds to the growing list of brain areas, namely the lateral ganglionic eminence, hippocampus and

the cerebellum, where apoptosis is observed before neurons migrate to their final destination [115, 165].

The neurotrophic theory describes the survival of neurons to be dependent on access to specific NTs supplied by their targets. Based on this definition, cells in the p0 medial SVZ should be exempt from developmental apoptosis, because they are located at their birth site, and have yet to undergo synaptogenesis with a target. Despite these considerations, the majority of dying cells in the p0 medial SVZ are immature, undifferentiated neurons and blockade of TrkB signaling results in increased apoptosis in the region. It appears that cells in the p0 medial SVZ are dependent on TrkB signaling for their survival, prior to their migration out of the area. Next steps should be to determine if the excess apoptotic cells in the p0 brains treated with TrkB IgG are immature neurons, as well as to determine the identity and source of the TrkB receptor ligand.

p7 SVZdl. One last point to consider is that in the context of the p7 SVZdl, the major cell type undergoing apoptosis was unable to be identified. Other groups studying the neonatal SVZ have encountered the same challenge. The Levison group was also unable to classify the majority of apoptotic cells when characterizing the SVZdl during the first and second postnatal week [118]. As mentioned earlier in the discussion section of chapter 3, the apoptosis kinetics in the p7 SVZdl may be faster compared to the p0 medial SVZ. The cell specific antigens could either be more sensitive to degradation or degraded at a faster rate in the p7 SVZdl than in the p0 medial SVZ. If this is proven true, then it might render the cell marker antigens unavailable for antibody

co-detection with pH2ax in the p7 SVZdl and reveal another inherent difference between the neonatal SVZ subregions.

6.1.3 Neurotrophin Signaling Regulates Cell Survival in the Developing SVZ

My data indicate that TrkB receptor signaling regulates cell survival in the p0 medial SVZ. The role of TrkB signaling in the SVZ had been mostly studied in regard to its influence on proliferation and migration in the adult [158, 160, 166]. The results here show that when TrkB signaling is disrupted in the p0 medial SVZ, the subregion where apoptosis is at its highest, there is an increase in the number of detected apoptotic cells.

Despite the significant apoptotic increase in the p0 medial SVZ of TrkB IgG injected brains versus Goat IgG controls, one would have still expected a greater increase in pH2ax+ cells, if TrkB signaling was an essential component in survival. One explanation for the limited increase in pH2ax+ cells in the TrkB IgG injected brains concerns the length of time TrkB IgG was available in the medial SVZ to block TrkB receptors. Brains were assessed one day after injection, and drug diffusion cannot be ruled out. Collecting and analyzing brains at earlier time points from the time of injection will determine if TrkB IgG availability influenced the lower than expected pH2ax+ cell number increase in the medial SVZ.

The findings regarding TrkB signaling and survival in the p0 medial SVZ could be linked with the demonstrated role of Bim in the same subregion at p0. It is worth investigating if TrkB receptor inactivity acts as a signal to initiate the intrinsic apoptotic cascade, starting with the activation of the BH3-only protein Bim. The results from this study would link the intercellular and intracellular mechanism of neonatal SVZ apoptosis,

which had been explored in the thesis. A relationship between NT signaling and BH3-only protein activation in apoptosis is not unprecedented. Sympathetic neurons exposed to NGF withdrawal *in vitro* show a subsequent increase in Bim mRNA and protein, with Bim elimination conferring transient protection from apoptosis [135, 145].

TrkB signaling has been shown to be regulate survival in the p0 medial SVZ, but the specific details of the process still await further characterization. One salient issue is identifying the NT/NTs activating TrkB receptors in the p0 medial SVZ. TrkB is activated by BDNF and NT-4, and both have been shown to be present in the adult SVZ and choroid plexus respectively [158, 160]. The next steps would be to determine the expression of both NTs in the neonatal SVZ, as well as their source.

The remaining points of discussion in this section concern the investigation of whether the effects of TrkB signaling extend to other subregions of the p0 and p7 SVZ, as well as the role of other NT receptors in the neonatal SVZ. The experiments concerning cell survival dependent TrkB signaling in the neonatal SVZ were performed solely in the p0 medial subregion. Stereotactic injection of TrkB blocking antibodies into the other p0 and p7 SVZ subregions followed by assessment for apoptotic cells would determine if the effects of TrkB signaling can be generalized to other SVZ subregions and later developmental time points.

The use of TrkB, BDNF and NT-4 knockout mice could be used as a tool, in addition to the stereotactic injection of blocking antibodies and inhibitors into the SVZ, for the investigation of TrkB's role on apoptosis in the SVZ subregions at different time points. The caveat with the use of TrkB and BDNF knockouts is that the majority of the

mice die between p2 to p14 [98, 158]. Even if a neuronal specific knockout were employed, there is always the possibility for indirect phenotypic effects stemming from the removal of TrkB, BDNF or NT-4 in other brain regions outside of the SVZ. At the time of writing there is no information on a TrkB, BDNF or NT-4 specific knockout for the entire SVZ.

Keeping the limitations of results gathered from knockouts in mind, TrkB specific removal in striatal neurons has shown p0 lateral SVZ cell survival dependence on TrkB activation [115]. Other SVZ subregions still await assessment. In terms of TrkB function at later time points, if experiments in the adult show a conserved influence of TrkB signaling, then TrkB will be necessary for cell survival in the p7 SVZ and later temporal periods [167].

Finally, the results from these experiments do not exclude the potential involvement of other NTs and their partner receptors in cell survival in the neonatal SVZ. Experiments looking at the expression of TrkA and TrkC and their ligands NGF and NT3, in the neonatal SVZ along with functional experiments blocking receptor signaling would provide an account of the role of these NTs, if any at all.

6.2 Implications for Therapeutic Strategies

Studying SVZ biology during development is important for a more holistic scientific understanding of this proliferative brain region, and the process of postnatal neurogenesis. In addition to this is the potential for findings on SVZ developmental apoptosis to be translated into practical clinical applications. The manipulation of

neonatal SVZ derived glia and neuronal numbers may prove useful for controlling the spread of gliomas, as well as increasing cell survival in cell transplant therapies.

Gliomas, which include astrocytomas, glioblastomas, and oligodendrogliomas, are the most commonly observed brain tumors in adults, and the primary cause of cancer death in children [168, 169]. The relationship between the adult SVZ germinal zone and the incidences of gliomas has long been studied [169-171]. A number of glioma cases are periventricular or contiguous with the SVZ [169]. Additionally, glioblastomas that contact the SVZ and migrate into the cortex are more likely than those that do not to be multifocal and recurrent [171]. Since gliomas can arise from either stem cells or mature glial cells and the SVZ is a site for both, it is worth exploring whether *in vivo* SVZ apoptosis mechanisms can be translated into promoting glial apoptosis in the context of gliomas.

Thesis chapter 3 reported both types of glial cells undergo cell death in the p0 medial SVZ and p7 SVZdl. The effects of Bim, PUMA and TrkB signaling on regional cell death were tested. I found that Bim expression is necessary for apoptosis and TrkB signaling for cell survival in the p0 medial SVZ. Bim is not expressed in glial cells, however there is some evidence for astrocytes to possess TrkB receptors [137, 158]. Future studies should investigate whether glia, in addition to immature neurons, comprise the neonatal cell population undergoing apoptosis in response to TrkB receptor blockade. If data gathered from the neonatal SVZ show glia are influenced by TrkB signaling, it might provide a mechanism for targeting these cells for elimination in gliomas.

Methods that increase SVZ cell survival could also hold important roles therapeutically as well. The widely heralded clinical application of SVZ research was for its use in cell transplant therapies [172-174]. Cell transplant experiments using perinatal SVZ cells have already been performed [175]. One challenge with implementing this technique is the cell death that affects the transplanted stem cell and neural progenitor population [176]. Ongoing research is aimed at determining the environmental cues and cellular signaling pathways that increase the survival of these cells [176]. TrkB signaling and decreased Bim expression have been shown to diminish apoptosis incidents in the p0 medial SVZ. Determining whether the manipulation of TrkB signaling or Bim expression could affect the survival of SVZ cell transplants is an important next step.

Coupling cell transplant therapy with the brains own healing properties may prove powerful in combating stroke and other conditions characterized by pathological apoptosis. Reports have shown that the brain possesses an endogenous self-repair mechanism through the cellular processes occurring in the adult SVZ [177-179]. It is not known if the same processes occur in the neonate. As a result of ischemia, Huntington's Disease (HD) or Parkinson's Disease (PD), there is an increase in SVZ proliferation and migration of SVZ neuroblasts to the affected brain region [177-179].

Despite this optimistic finding is the reality that very few of the neuroblasts that migrate out of the adult SVZ survive at their final destination [168, 179]. The results reported in the thesis chronicle the neonatal SVZ, but it is worth investigating whether the factors that raise the susceptibility of neuroblasts to apoptosis in the developing SVZ (i.e. Bim expression, TrkB signaling disruption) may also be in effect with the SVZ

derived neuroblasts that migrate to areas of neuronal loss in ischemia, HD and PD. It has already been shown that adult mouse models of neonatal hypoxic-ischemic brain injury infused with a combination of BDNF and epidermal growth factor into the ventricle display increased neuroblast survival at the injury site compared to controls [180].

6.3 Closing Statement

The presented thesis work is a study of apoptosis affecting the neonatal SVZ. These experiments are the first to quantify apoptosis in all neonatal SVZ subregions at two developmental time points, the involvement of Bim in the SVZ apoptotic process and the dependence of the medial SVZ subregion on TrkB signaling for cellular survival. The collected results have also raised intriguing questions concerning elements of the long established NT theory, by showing the dependence of a premigratory immature neuronal SVZ population on NT signaling, and the resulting apoptosis fallout from the lack of TrkB activation. The findings from this work extend the limited information surrounding developmental apoptosis in the immature SVZ, and hold the potential to aid future translational studies in cell transplantation and therapies to treat gliomas.

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